LIPOIDAL SPECIES IN OCULAR SPOILATION PROCESSES.

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SUMMARY OF THESIS.

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Tear component deposition onto contact lenses is termed 'spoilation' and occurs due to the interaction of synthetic polymers with their biological fluid environment. Spoilation phenomena alter the physico-chemical properties of hydrophilic contact lenses, diminishing the optical properties of the lens, causing discomfort and complications for the wearer. Eventually these alterations render the lens unwearable. The primary aim of this interdisciplinary study was to develop analytical techniques capable of analysing the minute quantities of biological deposition involved, in particular the lipid fraction. Prior to this work such techniques were unavailable for single contact lenses. It is envisaged that these investigations will further the understanding of this biological interfacial conversion. Two main analytical techniques were developed; an high performance liquid chromatography (HPLC) technique and fluorescence spectrofluorimetry. The HPLC method allows analysis of a single contact lens and provided previously unavailable valuable information about variations in the lipid profiles of deposited contact lenses and patient tear films. Fluorescence spectrophotofluorimetry is a sensitive non-destructive technique for observing changes in the fluorescence intensity of biological components on contact lenses. The progression and deposition of tear materials can be monitored and assessed for both *in vivo* and *in vitro* spoiled lenses using this technique. An improved *in vitro* model which is comparable to tears and chemically mimics ocular spoilation was also developed. This model allows the controlled study of extrinsic factors and hydrogel compositions.

These studies show that unsaturated tear lipids, probably unsaturated fatty acids, are involved in the interfacial conversion of hydrogel lenses, rendering them incompatible with the ocular microenvironment. Lipid interaction with the lens surface then facilitates secondary deposition of other tear components. Interaction, exchange and immobilisation (by polymerisation) of the lipid layer appears to occur before the final and rapid growth of more complex, insoluble discrete deposits, sometimes called 'white spots'.

Keywords: tear lipids, high performance liquid chromatography, fluorescence spectrofluorimetry, *in vitro* ocular spoilation model.

To my parents and Ben.

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ABBREVIATIONS.

Ca^{2+}	calcium
Cl^-	chloride
CIMS	chemical ionisation mass spectrometry
CH_3Cl	chloromethane
$CHCl_3$	trichloromethane
CH_3OH	methanol
CCl_4	carbon tetrachloride
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EWC	equilibrium water content
FCS	foetal calf serum
GLC	gas-liquid chromatography
γ_p	polar component of surface energy
γ_d	dispersive component of surface energy
γ_t	total surface energy
HCL	hydrochloric acid
HCO_3^-	hydrogen carbonate
HEMA	2-hydroxyethyl methacrylate
HPLC	high performance liquid chromatography
H_2S	hydrogen sulphide
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G

ABBREVIATIONS.(continued)

IgM	immunoglobulin M
K^+	potassium
Mg^{2+}	magnesium
MA	methacrylic acid
MA01	0.1% methacrylic acid
MA02	0.2% methacrylic acid
MA05	0.5% methacrylic acid
MMA	methylmethacrylate
Na^+	sodium
NH_4OH	ammonium hydroxide
PAS	performic acid Schiff
PBS	phosphate buffered saline
PFAS	performic acid Schiff
PO_4^-	phosphate
R_f	distance moved by solute / distance moved by solvent
Rt	retention time
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope
THF	tetrahydrofluoran
TLC	thin layer chromatography
UV	ultra-violet
v/v	volume per volume
w/v	weight per volume
VP	vinyl pyrrolidone

CHAPTER 1.

Introduction : Overview of the nature of the problem.

1.1. Introduction.

Since the pioneering work of Wichterle and Lim [1] polymers have been extensively employed for a variety of biomedical applications. These applications include cardiovascular devices, burn dressings, artificial joint replacements, drug delivery systems and contact lenses [2-5]. Such implanted materials are termed biomaterials and interface with the biological tissue and fluid environment of the patient for significant lengths of time. As a result of implantation an interaction occurs between the biomaterial and it's biological fluid environment. The interaction between the biomaterial and the tissue is important in both the treatment of the patient and in the success of the biomaterial. For this co-existence to be successful the biomaterial must be biocompatible. Biocompatibility depends on complex, and as yet, not fully understood chemical and physical interactions between the implanted biomaterial and the biological fluid environment. A fully biocompatible material is therefore one which does not adversely or significantly affect the biological system or the biological phase adversely or significantly affect the biomaterial [6].

A problem, however, arises due to the interaction of these synthetic biomaterials and their biological environment. A rapid change occurs due to this interaction, which ultimately deposits a layer out of the biological system onto the biomaterial. The interaction process between these synthetic implants and the biological environment is termed biological interfacial conversion. Thus, an adsorbed layer will then influence any further interaction with the surrounding environment [7]. The underlying process is common to many of the applications of biomaterials previously mentioned. The deposition of tear components onto contact lenses, the clotting of blood at foreign surfaces [8], the formation of dental plaque, marine fouling and fibrous encapsulation of implants are all examples of this

underlying phenomenon. A similar phenomenon is also encountered during '*in vitro*' cell culture experiments [9].

The study of these initial reactions to biomaterials *in vivo* presents many problems principally because they are difficult to observe and monitor. The majority of biocompatiblity studies have involved implanting the biomaterial into a suitable host and examining the prosthesis on removal at a later date. The problem with this process is the trauma of surgery involved. It is, therefore impossible to study in this way those initial biological responses which govern the ultimate fate of the biomaterial. However, one successful *in vivo* model which is available to study this interfacial conversion is the interaction between the ocular environment and the contact lens. The eye is an easily accessible site allowing insertion and removal of the contact lens after a suitable period of time, without any trauma to the wearer, for examination. The tear fluid, although a complex biological fluid, is simpler than, but similar to blood (table 1.1). It contains none of the cellular components and lacks certain other species e.g. platelets, clotting factors which further complicate the study of the blood contact devices. These factors have implications which will be considered later, for the usefulness of the eye as a body site for spoilation studies.

The interaction between the ocular environment and the contact lens leads to deposition of tear components. This process is termed 'spoilation' and covers a range of phenomena which cause the physical and chemical properties of the hydrophilic contact lens to be changed. These changes take the form of a variety of deposits and coatings which diminish the optical properties of the lens, leading to discomfort and complications

for the wearer [10,11]. Eventually these alterations will render the lens unwearable.

Table 1.1:- Comparison of some blood and tear components.

Component	Tears (mg/dl)	Blood (mg/dl)
Albumin	104-150,390-394 [12-15]	2200-5500 [16-19]
IgA	17.0-24.2 [20-27]	140-420 [16]
IgG	< 0.1-14.0 [19,21-24,27,28]	600-1700 [16,21]
IgM	0.31-<5 [21,22,27,28]	50-190 [16,21]
IgD	< 1.0 [21]	3-40 [16,21]
IgE	0.0026-0.02 [21,29]	0.0052-0.16 [16,21,29]
Total globulins	275	410-1740 [16-18]
Lysozyme	65-215 [12,19,26,29,30]	
Lactoferrin	81-209 [19,28,31]	
Total protein	652-800 [12,21]	6500-6980 [21,32]
Cholesterol	8-32 [33]	
Total lipids	196-240 [12]	285-675 [16]
HCO_3^-	26 [30,34]	21-30 [14,16,20,30]
Cl^-	85-456 [14,30,36-38]	98-110 [14,16,20,30,35]
K^+	15-137 [12,14,18,30,38-40]	3.8-5.4 [14,16,30]
Na^+	142-354 [12,14,30,37,38]	132-150 [14,16,30,41]
Mg^{2+}	0.86-1.3 [12,29]	1.6-3.4 [16,29,41]
Ca^{2+}	2.6-7.6 [12,29,41,42]	5.0-5.6 [16,41]
PO_4^-	1.01-1.12 [12,43]	0.9-2.7 [16,41]

The frequent use of cleaning products is therefore, necessitated in an attempt to prevent deposit formation, but little success has been achieved in the inhibition and removal of such deposits [44,45]. Cleaning care is also very expensive and may even cost as much per year as the actual lenses themselves.

Contact lenses may be divided into two broad groups based on material content. The groups are (i) hard lenses typically composed of methyl methacrylate and its copolymers with siloxy-methacrylates, (so called rigid gas permeable lenses) and (ii) soft hydrogel lenses which are typically based on hydroxyethyl methacrylate or variations of this monomer with vinyl pyrrolidone and methyl methacrylate. Synthetic elastomer lenses such as those based on silicone rubber (polydimethylsiloxane) are also potentially useful, but have little place in commercial practice. The underlying material science that governs the usefulness in contact lens applications depends upon a combination of features such as oxygen permeability, mechanical properties and surface properties. Detailed consideration of these factors is outside the scope of this introduction, but they have been adequately reviewed elsewhere [46,47,48].

Currently there is no well founded mechanism or explanation for these deposition phenomena. Despite extensive work and the volume of literature available on hydrogel lens spoilation [49-59], other manifestations of this spoilation process [60-69], and adsorption studies using protein and lipids [70-84], very little is known about the initial events which cause the changes to occur. Such explanations as are advanced are extrapolated from studies related to blood compatibility and usually imply a dominant role for protein adsorption. Although protein adsorption is a rapid process in any environment the absence

in tears of blood clotting proteins suggests that caution should be exercised in making this extrapolation.

Before embarking on studies into this biological interfacial conversion process which leads to spoilation using the contact lens model, it is necessary to examine the ocular environment and the manifestations of spoilation produced by the interaction between this environment and the contact lens.

1.2. Ocular environment.

The composition of the tear fluid as it exists in the conjunctival fornix and in the precorneal tear film is of a complicated nature. The precorneal tear film is a physically inhomogeneous system produced by the lacrimal glands, the accessory lacrimal glands, goblet cells of the conjunctiva and the Meibomian glands of the lid margin [85]. Other small glands are also involved [14].

Tears serve a number of functions [86,87]:-

1. They act as the anterior refracting surface of the eye, filling in irregularities in the corneal epithelium.
2. They flush away noxious substances from the eye.
3. They transport metabolic products (particularly O_2 and CO_2) to and from the corneal and conjunctival epithelium.
4. They provide a pathway for white blood cells to the central avascular cornea in the event of corneal trauma.
5. They provide lubrication between the lids and the ocular surface.

6. They contain antibacterial substances and antibodies.

7. They maintain the corneal stromal deturgescence and the lower corneal tissue temperature in relation to the iris by water evaporation.

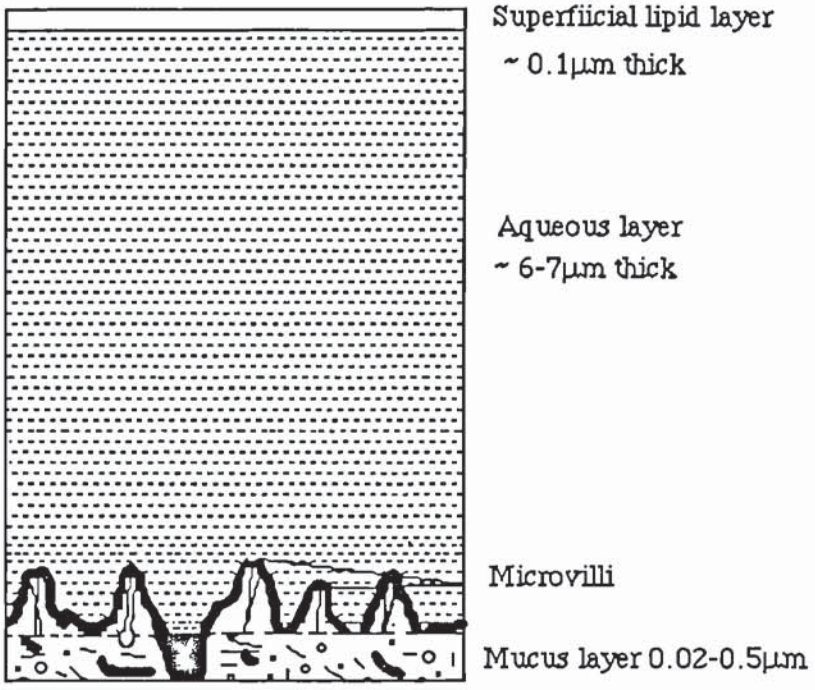
8. They serve as an exit pathway for desquamated epithelial cells and other debris.

1.2.1. Tear Film physical characteristics.

The precorneal tear film is a complex layer of fluid with a thickness of 5-10μm[88,89]. The originally accepted scheme for the tear film was suggested by Wolff [90] to be composed of a three-layered structure; consisting of a superficial oily (lipid) layer, a middle aqueous layer and a deep mucoid layer. The superficial lipid layer is a thin (0.1-0.2μm[85,91]) film of Meibomian lipid at the air/tear interface and covers the aqueous layer (6-7μm [88]), which contains proteins [85] metabolites [92], electrolytes [35-40] and mucin [93,94]. The adsorbed mucoid layer (0.02-0.04μm [85,98,99]) on the epithelial surface of the cornea, rendering it hydrophilic and allowing the tears to spread over the surface after blinking (figure 1.1).

The volume of the tear fluid is about 5-10μl [38,54,55] with a pH of 7.4 (7.3-7.7), refractive index of 1.357 [100] and a viscosity of 1.05-1.40cP. The osmotic pressure of the tear film is 300mOsm/l which is approximately equal to that of 0.9% sodium chloride solution (289 mOsm/l). The secretion rate of human tears varies with age and sex [30].

Figure 1.1:- Schematic diagram of the tear film.



1.2.2. Tear composition.

Due to the highly complex nature of the tear film and the number of sources which secrete it, the method of collection for analysis is crucial to it's quantification. The method of collection therefore has a large effect on the results obtained [85,101]. This is particularly true of absorbent materials and those which may cause corneal irritation. There is also a wide variation in the composition of the tear fluid between individuals, which also presents problems when analysing the pooled samples required due to the minute quantities which can be extracted from the eye.

1.2.3. Superficial lipid layer.

The superficial lipid layer, as previously stated, is the outermost layer of the tear film. Most of the lipids in this layer originate from the Meibomian glands which are

embedded in the tarsal plates in each lid [14,91]. Under normal circumstances the action of blinking is sufficient to express the lipids onto the aqueous layer. The Meibomian glands bear anatomic, histologic and histochemical similarities to the sebaceous glands of the skin, although the composition of the glands is different [102]. The thickness of the lipid layer varies, when the eye is open it has a thickness of about 0.1μm whereas on compression by the eye lids during closure it is 100μm [103]. When the eye is opened, the compressed lipid layer rapidly expands across the cornea. This monolayer of lipid has a stable consistency, which protects the aqueous layer by retarding evaporation [104-106].

The composition of the Meibomian lipids in humans varies considerably between individuals, although the major classes of wax esters, cholesterol esters and triglycerides are always present [107-109]. Analysis of the Meibomian lipids demonstrate the presence of of all possible lipid classes, mainly hydrocarbons, wax esters, cholesterol esters, triglycerides and in lesser quantities diglycerides, monoglycerides, free fatty acids, free cholesterol and phospholipid [110-112]. Tiffany [107,113] concluded that no single composition exists for human Meibomian gland oil and that results obtained from pooled material from many individuals maybe misleading as an indicator of ocular function. Similar lipid compositions are also found in other biological fluids e.g. saliva, sebum [35,114-116].

1.2.4. Aqueous layer.

The middle aqueous layer is secreted by the lacrimal glands and the glands of Krause and Wolfring. This layer is 6-7μm thick and contains numerous electrolytes [117], vitamins, proteins, metabolites, enzymes, vitamins, water, various organic acids and antiproteinases [118].

Water; The tears contain 98.2% water [34]. The rate of evaporation of water from the tear film is between 8 x 10^{-7}gmcm^{-2}sec^{-1} and 10.1 x 10^{-7}gmcm^{-2}sec^{-1} [119,120] and is controlled by the superficial lipid layer.

Electrolytes; The positively charged electrolytes in tears are mainly sodium and potassium and the negatively charged ions are chloride and bicarbonate [121,122]. The tears also contain calcium [123] and magnesium [85].

Table 1.2:- Summary of electrolyte concentrations in tears.

	Concentration in mmole/l					
	Na^+	K^+	Ca^{2+}	Cl^-	HCO_3^-	Reference
Tears	120-170	6-26		118-138		38
	80-161	6-36		103-130		36,39,92
	145	24		120	26	14
	134-170	26-42	0.5-1.1	120-135		12
			0.4-0.8			124
			0.4-1.1			125,126
			0.3-2.0			127
Serum	140	4.5	2.5	100	30	Average of normal

Manganese is also present in tears and is believed to be involved in emotional tears, as this ion has an important function in affecting mood [128]. Bicarbonate ions maintain a stable ocular tear pH by providing a buffering system [129].

Proteins; Tears contain a number of proteins. The exact protein composition and their concentrations is dependent on the method of collection [85]. The major proteins found include tear albumin, specific tear pre-albumin, globulins, lysozyme, lactoferrin, transferrin, caeruloplasmin, immunoglobulins A, G and E, glycoproteins and complement. Many of these proteins serve a function in preventing microbial invasion and oxidative damage to the surface of the eye [130]. Tear specific pre-albumin is a binding protein which is concerned with retinol carriage in the tears and is important for the delivery of vitamin A to the corneal epithelium [131]. Lysozyme is a bacteriolytic protein which is important in the defensive mechanism of the eye and also interacts with immunoglobulins A and G. The main immunoglobulin found in the eye is immunoglobulin A (IgA) [23,26,132] with small concentrations of immunoglobulins G and E. Secretory IgA coats bacteria rendering them mucophilic and preventing them attaching to the epithelium. The other immunoglobulins are involved in complement mediated micro-organism lysis. Complement is also involved in bacterial oponisation, chemotaxis, and micro-organism phagocytosis [133]. Lactoferrin is a potent chelating agent which deprives certain bacteria of essential iron and is also involved in the anti-microbial processes previously mentioned. Caeruloplasmin, is a copper-carrying protein which is also a ferroxidase and is involved in free-radical oxidation.

Metabolites; The metabolites contained in the eye include pyruvate, glucose, lactate, urea, catecholamines (dopamine, adrenaline, noradrenaline and dopa), histamine, endorphins and prostaglandins.

Enzymes; A number of enzymes are found in tears. These include glycolytic enzymes, tricarboxylic acid cycle enzymes, lactate dehydrogenase, lysosomal enzymes, amylase, peroxidase, plasminogen activator and collagenase. These enzymes are involved in energy production and the maintenance of the corneal epithelium.

Antiproteinases; The antiproteinases, inhibitors of proteases, are present in tears in much lower concentrations than in the plasma. They include α_1-antitypsin, α_1-anti-chymotrypsin, inter-α-trypsin inhibitor and α_2-macroglobulin.

Vitamins; The vitamins present in the eye include Vitamin A and Vitamin C (ascorbic acid).

1.2.5. Mucoid layer.

The mucoid layer is in direct contact with the anterior surface of the corneal epithelium. This layer is rich in glycoproteins and is derived from the conjunctival goblet cells. The glycoproteins are carbohydrate-protein complexes characterised by the presence of hexosamines, hexoses and sialic acid[134,135]. Holly and Lemp (1971) [136] suggested that the primary role of these conjunctival glycoproteins is to alter the low energy corneal surface into a higher energy surface via adsorption. This effect combined with increased affinity toward water and the lowering of the surface tension of the tears is sufficient to achieve the complete wetting of the corneal surface. Mucus also lubricates, hydrates and

protects the underlying corneal epithelium. Mucins are more surface active than simple proteins such as albumin or serum globulins. It is capable of interacting with lipids increasing their film pressure, of masking lipid hydrophobicity and efficiently removing lipid contamination, without interfering with cell membrane integrity [137]. Tear mucins (mucous glycoproteins) are also responsible for the high relative viscosity of tears [138]. Mucus is also important in the defence mechanism of the eye.

Thus the tear fluid is a highly complex interacting biological mixture. The contact lens when placed in this ocular environment contacts directly with the anterior corneal surface. The outer lens surface interacts with the lids, especially during blinking [139]. The contact lens is therefore completely surrounded by this complex biological fluid and is rapidly coated with tear film derived material upon insertion into the eye. Interaction with the tear film and lens coating then allows the process of interfacial conversion to occur, producing spoilation. A brief description of the manifestations of this spoilation will now be given.

1.3. Manifestations of spoilation phenomena in Hydrogels: The position at the outset of this work.

Spoilation of hydrogels is manifested in many forms. These vary from lens to lens and from one patient to another. The reported clinical incidence of contact lens spoilation by the wearer varies. The majority of patients show spoilage after 3-6 months of daily extended wear, although it may occur as early as 48 hours of wear [10]. The factors which contribute to the deposition varies widely between patients and care regimes used.

A variety of organic, inorganic and mixed deposits adhere to the lens surface. Due to this variation in types of spoilation, it is convenient to divide the observed types of spoilation into different classes. One such classification is based on the grouping together of the various related types of clinically observed phenomena:- (e.g. [49])

1. Complex deposits: discrete elevated deposits (white spots), particles.
2. Lens coatings: proteinaceous films, specific calcium deposits, granular deposits, inorganic films.
3. Microbial deposits: fungal and bacterial deposits.
4. Extrinsic factors: cosmetics, dust, metallic particles from the atmosphere.

This is, however, an artificial classification. There is considerable overlap between classes and as a result systematic analyses of the results of spoilation are rarely undertaken in conjunction with clinical reports relating to incidence.

The discrete elevated deposits or 'white spots' are most chemically complex manifestations of ocular incompatibility. They are also referred to in the literature as mucoprotein-lipid and mucopolysaccharide deposits. These deposits are found on the anterior surface of the lens and have no specific site or orientation on the lens surface. These deposits seem to be found in groups rather than independently and they are often seen concentrated in areas on the lenses [140].

Their morphology appears to be formed by the successive laying down of globular structures tightly packed together as a complex multi-layered structure [141]. A more detailed analysis of this complex structure shows three distinctive, yet interactive

sub-layers[49,142,147].

1. A basal layer is formed, which directly interfaces with the lens matrix. This interfacial layer is vital for deposit formation. The basal layer may exist without the deposit, but the deposit can not exist without the basal layer.
2. The secondary layer within a deposit constitutes the bulk of the deposit. It is ellipsoidal dome-shaped structure with numerous lobular sub-units.
3. The tertiary layer is a transparent film-like coating which is complex and multi-nodular. This overall morphology appears unaltered by variation in the surface properties of the lens, wear protocol or application of the device.

The chemical composition of the deposits is very complex and variations may be dependent upon the quality and sensitivity of the analytical techniques used. Tear solutes include proteins (principally albumin, globulins, lysozyme but many others), amino acids, mucin, glycoproteins, glucose and lipids (including phospholipids, neutral lipids, fatty acids, cholesterol and its esters), calcium, potassium, chloride, bicarbonate, phosphate and urea. Although individual variations do exist especially in the mucoprotein content and in the Meibomian secretions. These deposits are believed to be composed of a high proportion of lipids. Some analytical techniques detected cholesterol, cholesterol esters, triglycerides, monoglycerides, fatty acids and fatty alcohols [142-145]. Whereas the presence of diglycerides, triglycerides, cholesterol esters and cholesterol were not detected by other techniques [146]. Calcium, which was originally believed to be a major constituent, has now been shown to be a variable component [147]. Calcium is however, often present in the larger deposits [146,148]. The vital role of micro-organisms has also been shown to be

minimal, as not all spoilt lenses have microbes present on them [14].

The lipids present in the deposits are naturally occurring in tears [85,101,149]. There appears to be some selective adsorption of some of the components. Triglycerides for example, have been shown at a higher relative concentration on the contact lens than in tears [148].

This spoilation mechanism is still unclear. Several factors are believed to accelerate deposition. These include dryness and stress on the lens surface [150-152] and a build-up of lipids secreted from the Meibomian glands [153]. It has also been suggested that the use of diuretics, incomplete blinking, potassium tear deficiency, high alcohol consumption and Meibomian gland abnormalities are linked to deposition of lipid on the anterior surface of the contact lens [154-160]. Defects or faults in the lens surface, which may occur during manufacture or arise during the life of the lens, may also increase deposit formation. These defects may arise from the use of surfactants, enzymatic preparations and chemically oxidative cleaning regimes to alleviate deposit formation.

It is with the absence of appropriate and sufficiently sensitive analytical techniques that this thesis is principally concerned. There are conflicting reports in the literature on the actual mechanism of the deposition process [8,10,53], but in the absence of analytical techniques that enable the early stages of spoilation to be studied, they are at best, speculative.

A major class of spoilation involves surface films, coatings and plaques. The proteinaceous films appear as thin semi-opaque, white superficial layers of denatured

protein [161]. The general accumulation of protein films on hydrophilic contact lenses leads to an increase in the haziness and rugosity of the lens. There are conflicting reports on their composition [161-163]. They are attributed to mucoproteins, albumin, globulins, glycoproteins, lysozyme and mucin [164-167]. There is also a correlation between the rate of deposition and tear break-up time. Tear break-up time produces dry spots as previously described. These hydrophobic areas then being favourable for deposit formation. The incidence of proteinaceous films is also higher on thermally disinfected daily wear lenses.

Inorganic films appear to be similar to the proteinaceous films, but are composed of insoluble crystalline materials [168]. These may cause damage to the lens due to penetration of the lens matrix [169-171]. These films often have an observable protein cover. They are mainly calcium phosphate which is co-precipitated with protein. The main cause of such films appears to be due to precipitation and growth of calcium phosphate from tears. Tear chemistry of the wearer, tear production and poor blinking all contribute to this type of deposit. The presence of protein films may increase the formation of inorganic films and deposits due to its hydrophobic nature, which is more likely to dry out between blinks [172]. These films may well be composed of the thermodymically stable phase of calcium phosphate; hydroxyapatite, which is encountered in biological conditions. Once nucleated, hydroxyapatite will grow from slightly supersaturated solutions like tears. There may also be a link between hydrophilic lens calcification and the mechanism causing corneal calcification [173]. This mechanism is briefly, that the release of calcium and phosphorous from injured cells could increase the calcium-phosphate produced locally. This along with the release of cellular enzymes like alkaline phosphatase and adenosine triphosphatase may cause corneal calcification. This is due to the action of this enzymes on pyrophosphate

which they hydrolyse to phosphate. The pyrophosphates are naturally occurring inorganic compounds which act as physiological inhibitors of calcification in soft tissues.

Granular deposits are a special form of crystalline deposit. These are elevated white or translucent formations of variable size. There are several hypotheses for their formation. Hilbert et al [174] suggest that they arise from boiling unused lenses in undistilled water. Koetting [175] suggests that the crystalline form disrupts the polymer allowing tear components, normally too large to penetrate the matrix, to be incorporated into the deposit. Hilbert et al [174] further suggest that the crystallisation of some ionic components of the tear film may occur during drying out. The influence of drying out is further substantiated as these deposits occur on the exposed anterior lens surface [10]. This type of deposit is very dependent on the wearer's tear chemistry. These crystalline growths appear to nucleate on the lens surface into thin amorphous structures which grow further by calcium phosphate crystallisation from tears.

Specific inorganic calcium deposits also occur. Calcium carbonate deposits consist of crystalline growths with definite needle-like forms. If these cover a large area, they appear like a film. They may grow into the lens and be covered by a protein film. With continued wear they become insoluble and appear like multiple lens cannaliculi. Kleist [168] showed that these deposits are less frequent on thermally disinfected lenses. It was suggested by Kleist that these deposits arise while the lens is in the eye. Freiberg [176] showed that these deposits may be dissolved, if they were recently formed, by thermal disinfection.

Microbial spoilage is the most serious hazard of spoilation. The occurrence of this type of spoilation is between 24-30% of patients fitted with soft contact lenses Some harmless flora are expected on the lenses, as these occur naturally on the conjunctival sac. Micro-organisms can also be transmitted to the lens due to lens handling and inappropriate lens care regimes. Many species of fungi and yeasts have been identified on soft contact lenses including *Rodotonita* species; *Candida tropicales, fusanum* and *albicans; Asperigillus fumigeltus, niger* and *Penicillium* species These microbes however are rarely copious on the lens due to the eye's highly potent antimicrobial action described previously [177]. Colonisation of the lens may arise due to their enzymatic activity on the polymer causing degradation and providing an environment suitable for growth. The presence of other deposits also encourages microbial invasions as they provide suitable nutrients for growth [178].

Discolouration of contact lenses arises due to a variety of factors including nicotine from cigarettes, topical adrenaline and topical vasoconstrictors as well as tear components. Yellow or brown discolouration is believed to be due to granular particles deposited just below the lens surface which are similar to melanin and immobilised within the hydrogel matrix. This discolouration may also be aided by adenochrome in eye medications which may be taken up by the lens. Melanin production is also stimulated by nicotine and other polycyclic aromatic compounds present in tobacco smoke. Thus it is not a direct discolouration but a nicotine-stimulated biochemical mechanism. Yellow or yellow-green discolouration arises due to chlorhexidine, which is found in preserved saline. Grey discolouration occurs as a result of mercury deposition. The mercury is derived form the thiomerosal [179] found in many hydrogel lens care systems. These deposits are more

commonly found on thermally disinfected lenses utilizing thiomersal-preserved saline. The primary cause however, appears to be due to the re-use of preserved saline or cold disinfection solutions. As the contaminant load of these solutions increases with each re-use, the thiomersal decomposes to a greater extent and finally mercurial decomposition products begin to deposit in the lens matrix [180].

Many extrinsic factors contribute to the spoilage of soft contact lenses and are cited[10] in the literature. These include cosmetics, poor lens handling, airborne foreign bodies, dust, toxic fumes and metal particles.

The above review of the manifestations of spoilation shows the need for research into the biological interfacial conversion which causes deposition. Due to the lack of sufficiently sensitive analytical techniques to analyse the minute quantities of material which form the deposits, few systematic investigations have been made into these initial events. As previously stated above lipids are now believed to play a prominent role in this interfacial conversion process. This study is primarily concerned with this prominent role of lipids in ocular spoilation and their analysis, as there are a number of different lipid classes which may occur in tears. A brief overview of these will now be given here.

1.4. Lipids.

Lipid is a general term that describes substances that are relatively water insoluble[181,182] and extractable by non-polar solvents such as chloroform, benzene, petroleum ether and diethyl ether [183].

Lipids fall into two broad categories;- the simple (non-polar or neutral) lipids and the complex (polar) lipids [184]. The simple lipids contain only fatty acids and alcohol components. The alcohol component is usually glycerol, but may also be a long-chain alcohol or sterol. Examples of simple lipids include triglycerides (triacylglycerols), fatty acids, cholesterol, cholesterol esters, wax esters etc.. The complex lipids are amphoteric in that they contain both a hydrophobic domain and a hydrophilic region in the same molecule. The two main subdivisions of complex lipids are the phospholipids and sphingolipids. These lipid classes have a variety of important biological functions within the body and outside it, including structural components of membranes [185,186] storage and transport forms of metabolic fuels, as protective coatings of many organisms and as components for cell recognition [187], tissue immunity and species specificity.

The prominent lipid classes found in animal fluids and tissues maybe listed as follows:-

Fatty acids; long-chain aliphatic carboxylic acids which occur in the free state in trace amounts. They can be saturated (e.g. palmitic acid) or unsaturated (e.g. oleic acid).

Triglycerides; the hydroxyl group of the trihydric alcohol, glycerol, is esterified to a fatty acid e.g. tripalmitin. Mono- (e.g. monopalmitin) and di- (e.g dipalmitin) glycerides also exist, but in lesser quantities.

Cholesterol; the commonest member of a group of steroids with a tetracyclic ring system.

Cholesterol esters; cholesterol esterified to a variety of fatty acids e.g. cholesterol palmitate.

Wax esters; consist of fatty acids esterified to long-chain alcohols.

Phospholipids; denotes any lipid containing phosphoric acid as a mono- or di-ester e.g. phosphatidylcholine.

Sphingolipids; consist of a long-chain base of fatty acids and inorganic phosphates, carbohydrates or other complex organic compounds e.g. sphingomyelin.

Glycolipids; consist of lipid (fatty acid and glycerol) and carbohydrate moieties e.g. galactosyldiacylglycerol.

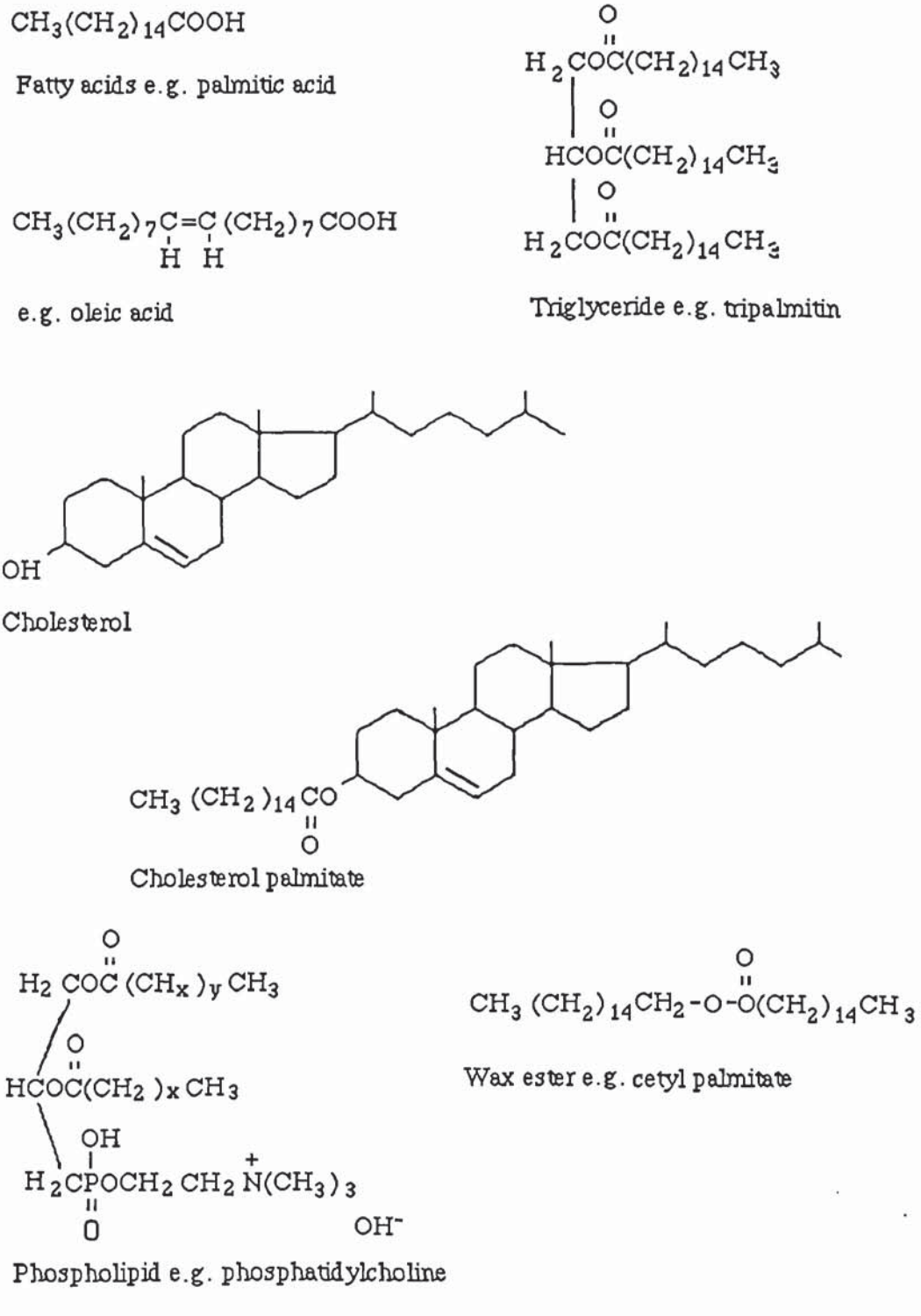
Lipoproteins; contain both lipid and protein moieties e.g. chylomicrons.

The structures of some of these lipids are shown in figure 2.1.

The ability of lipids to modify both hydrophilic and hydrophobic surfaces is due to their amphipathic nature. For example, with fatty acids the polar head groups may attach to hydrophilic surfaces or the 'hydrophobic' tail region may attach to a less polar surface rendering it more hydrophilic.

As well as their previously mentioned role in ocular spoilation lipids are also active in other polymer environments [188] and adsorb to other polymers. For example silicone heart valve poppets [189,190] and swelling and obstruction of silastic heart valve poppets[191]. Lipids have also been implicated in cell adhesion responses [192], for example in atherosclerosis where fatty lesions occur on the artery walls followed by ingrowth of cells, deposition of lipids and calcium causing hardening of the arteries. The role of lipids is also unclear in this disease. Thus any understanding of the primary interfacial conversion events involving lipids in the eye would be beneficial to other polymer implant applications in the body.

Figure 2.1:- The structure of some of the lipid classes described above.



1.5. Scope of this study.

The majority of the literature on soft contact lens intolerance has been based on the clinical manifestations of spoilation. Only a few analytical studies into these phenomena are recorded [58-61,142-148,167,193]. A previous study at Aston University has characterised such phenomena [140,142]. This study led to the belief that a common biological interfacial conversion event, or events, occurs reducing the biocompatibility of the contact lenses leading to spoilation, as previously explained. There is however, no mechanism or explanation for these initial events which cause spoilation manifestation especially white spot formation.

Before solid progress can be made in the understanding of the underlying mechanism that leads to spoilation of contact lenses the nature of the early biological interfacial conversion process must be determined. In order to achieve this analytical techniques must be developed allowing these early stages of spoilation to be followed. The main problem is the minute quantities of materials involved in the spoilation process on a single contact lens. Although there are several of techniques for analysing bulk quantities, like serum, urine etc.. Techniques applicable to the analysis of single contact lenses have not been developed. This is especially true in the case of lipids.

This study forms part of an investigation of all the appropriate biochemical species implicated in ocular spoilation. The aim was to develop suitably sensitive techniques to analyse and possibly identify the components involved in the initial events of biological interfacial conversion. Techniques to be developed were particularly concerned with the lipoidal species involved in these processes. The purpose of protein evaluation in this

thesis is mainly in the distribution of the lipid-protein separation. As the lipid is clearly important to the understanding of protein deposition i.e. does the protein deposition depend on the lens material or on the lipid interaction first with the lens surface and then protein. In the later stages of spoilation it is clearly the latter effect. In addition to this aim an *in vitro* model which can mimic ocular spoilation was also to be developed. This *in vitro* model should accelerate the spoilation of contact lenses and allowed the assessment of the particular components which need to be laid down to produce the clinical phenomena of ocular spoilation to be investigated. The model should also enable some extrinsic factors such as cleaning regimes and novel hydrogels which may have an effect on the spoilation process to be investigated.

CHAPTER 2.

Materials and experimental techniques.

2.1. Contact lenses.

Some of the contact lenses used in these studies were commercially available lenses, (table 2.1.).

Table 2.1: Principal components of commercially available lenses used during this study [46-48].

Lens	Composition	USAN nomenclature	Manufacture
Cooperthin	p(HEMA)	Polymacon	cast polymerised
Kelvin Eurothin	p(HEMA)	Polymacon	lathe cut
Permaflex 43	HEMA/VP/MMA	Tetrafilcon-A	cast polymerised
Permaflex 74	MMA/VP	Surfilcon-A	cast polymerised
Permalens	HEMA/VP/MA	Perfilcon-A	lathe cut
X-ten	Complex copolymer based on acrylamide monomer		lathe cut
Vistagel A	MMA/VP	Lidofilcon equivalent	lathe cut

HEMA = 2-hydroxyethyl methacrylate

MMA = methyl methacrylate

VP = vinyl pyrrolidone

MA = methacrylic acid

USAN = United States Approved Name.

Other materials were prepared and fabricated into lenses as required for individual study. Details are included, where appropriate in the the text.

2.2. Solvents.

Unless otherwise stated all solvents were obtained from Fisons and were of HPLC standard.

2.3. Histological stains.

All reagents employed in the histological analysis of lens deposits were of analytical grade and were obtained from B. D. H. Ltd. The compositions of fixation solutions and respective buffers are given in Appendix 1.

2.4. Sterilisation solutions.

The contact lenses used in *in vitro* studies (chapters 7 and 8) were sterilised with a commercially available cold disinfection regime (Septicon System).

2.5. Lipid standards.

All lipid standards were purchased from Sigma and were of at least 98% purity. A number of lipid standards were used. These included cholesterol linoleate, cholesterol oleate, cholesterol palmitate, triolein, trilaurin, trilinolein, arachidic acid, linoleic acid, linolenic acid, oleic acid, palmitic acid, stearic acid, L -α– phopshatidylinositol, L-α– phosphatidylcholine, L -α– phosphatidyl-L-serine, cholesterol and a lipid standard comprising triolein, oleic acid, cholesterol oleate and cholesterol.

2.6. Proteins.

All proteins were purchased from Sigma and were of the highest purity obtainable in each case.

2.7. Preparation of glassware.

All glassware was soaked for up to 24 hours in a phosphate free detergent (Decon Labs. Ltd.) after which time it was rinsed thoroughly in distilled water and heat dried. Prior to use the glassware was rinsed with a suitable solvent, either hexane or chloroform/methanol (2:1 v/v). To limit contamination gloves were worn at all stages of analysis.

2.8. Histology.

2.8.1. Lens fixation.

Formaldehyde, in the commercially available form of formalin was used as a primary fixative throughout this study. Formalin consists of free formaldehyde, methylene glycol and hydroxymethylene glycols [194,195]. Originally formaldehyde was thought to be unable to fix lipids [196], recent evidence however suggests it is capable of fixing lipids by reacting with unsaturated fatty acids [197]. 10% formaldehyde fixatives were used in the histological studies. The lenses were fixed for twelve hours in the fixative at room temperature. After fixation, the lens samples were thoroughly washed in distilled water prior to staining or sectioning.

2.8.2. Section cutting.

To section the lens samples were embedded in agar in a perspex mould (1cm x 1cm x 1cm) [147]. The lens was positioned horizontally. Once the agar block was set, it was

mounted vertically on the microtome (1320 Freezing Microtome, Leitz Ltd.) specimen stage and frozen. The sections, 10μm thick were cut using a carbide knife and floated out on distilled water, examined microscopically and finally stained.

2.8.3. Staining techniques.

The staining techniques were employed for the chemical identification and localisation of components in the contact lens deposits. The stain methodologies employed in this study are described in various texts [198,199]. Control sections were also treated in a similar manner to the spoiled lenses. The chemical specificity of the stains used was wide ranging; from general stains such as Oil Red O for lipids, to the highly specific Von Kossa calcium detection method.

2.8.4. Section mounting and examination.

Prior to mounting, all sections were washed in distilled water and then centrally positioned on glass slides. Specimens were then immersed in a drop of glycerin-albumin suspension, covered with a glass cover-slip and oven dried. Mounted sections were then examined microscopically.

2.9. Light microscopy.

Several types of microscopy were used during this study. These include brightfield, phase contrast, fluorescence and darkfield.

Brightfield is the 'normal' microscope. The specimens under this type of monochromatic illumination change the amplitude of light, so that the different areas appear

at different brightness.

Phase contrast microscopy is used to render transparent objects visible. This is achieved by converting the phase difference in the light passing through them into changes of wave amplitude. It occurs by interference with the light in the same field. Thus a very small phase difference, due to differing refractive indices are converted into clearly visible brightness differences.

Fluorescence microscopy is a sensitive method for measuring minute quantities of fluorescent material. The fluorescence intensity is proportional to the intensity of the incident light. Fluorescence occurs as a result of absorption of a photon of light which is followed by the emission of light of a longer wavelength (i.e. lower energy). Thus when a specimen is illuminated with an exciting wavelength of light and observed through a filter which excludes the exciting light, but transmits the fluorescence, components of the specimen which are difficult to detect can be made visual. A wavelength of 360nm was used unless otherwise stated throughout this work.

Darkfield microscopy is mainly for transparent objects whose structures are based on changes of the refractive index. These refractive changes particularly occur across the edges of a specimen. These edges are therefore, brightly illuminated against a dark background.

In this study contact lenses were examined under a Leitz Dialux 20 microscope with a Wild MPS 15/11 camera attached to allow photographs of the contact lenses to be taken.

The examinations of the lenses allowed the manifestations of soft contact lens spoilation to be observed. It enabled the integrity of worn and unworn lenses, the morphology, fluorescence and geological location of the deposits on and within the lenses to be assessed.

2.9.1 Light microscopy: Sample Preparation.

The contact lenses were stored in preserved saline solution after being cleaned using a cold regime to stop microbacterial contamination. The lenses were then only handled with clean forceps.

The contact lenses were mounted according to the method of R.W.J.Bowers [147]. After the microscope slide was cleaned with sodium dodecyl sulphate (SDS) and dried. The contact lens was mounted with preserved saline and flattened with a glass cover slip. This procedure allows the lens to be examined with minimum surface distortion, caused by dehydration and with only minor changes in focus. Lenses examined in this way can be observed repeatedly without any damage being incurred. Care is however necessary with extracted lenses as these tend to become brittle and prone to splitting.

2.10. Fluorescence spectroscopy.

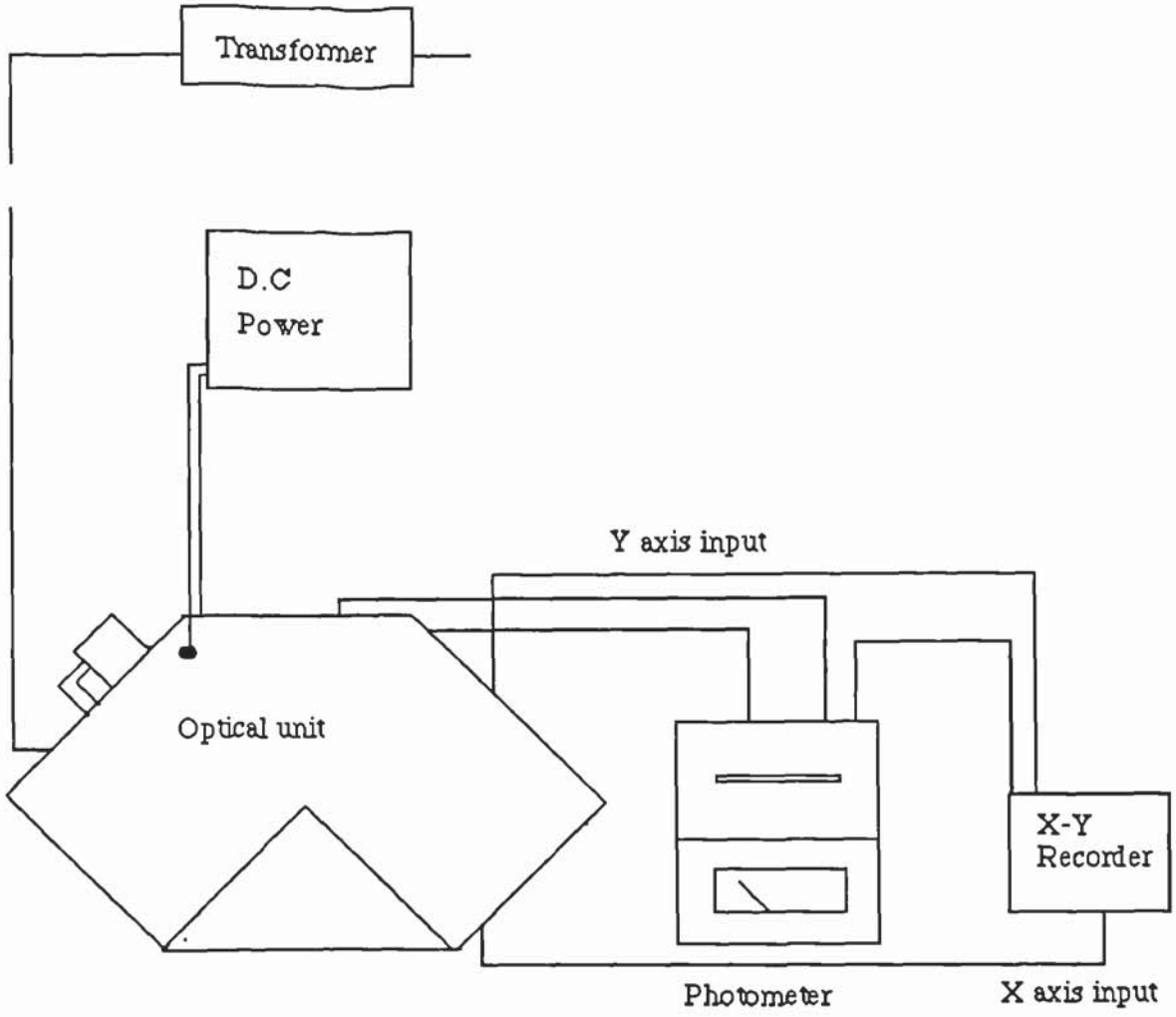
With some molecules, the absorption of a photon is followed by the emission of light from an electronically excited state. This light is of a longer wavelength (i.e. lower energy) than that of the adsorbed photon.

Fluorescence emission results from the return to the lower orbital of a paired

electron. In a singlet excited state, the electron in the higher energy orbital has the opposite spin orientation to the second electron in the lower orbital. These electrons are paired.

Substances which display significant fluorescence generally possess delocalised electrons present in conjugated double bonds. There are two types of fluorophore; intrinsic and extrinsic. Intrinsic fluorophores are contained within the macromolecules themselves e.g. proteins and lipids. Extrinsic fluorophores are added to the system, usually binding to one of the components e.g. fluorescein, fluorescamine [200-203]. A typical fluorescence spectrophotofluorimeter is shown in figure 2.1. In this study two fluorescence spectrophotofluorimeters, both Aminco-Bowman instruments, were used to non-destructively monitor the lipid and protein present on the lenses.

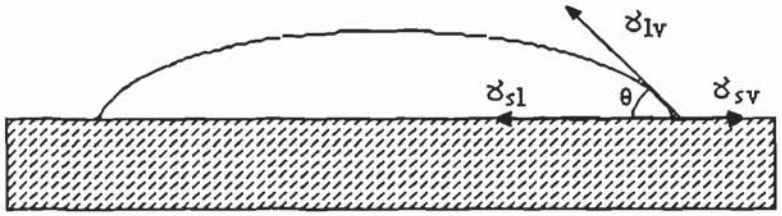
Figure 2.1:- A typical fluorescence spectrophotofluorimeter.



2.11. Contact angle measurement.

Contact angle measurements were considered of value in this study because these have been shown to be a useful technique to evaluate the surface energy and wettability of materials. Furthermore, contact angles can be easily measured and the principle behind the technique is based on the fact that most liquids in contact with a solid will spread. The extent of this spreading is determined by the balance of forces at the boundary line between liquid, solid and air, as shown in figure 2.2.

Figure 2.2:- The forces involved in determining the contact angle, θ, of a liquid drop on a surface.



The balance of forces outlined above may be further described by the following equation [204]:-

$$\text{Cos } \theta \cdot \gamma_{lv} = \gamma_{sv} - \gamma_{sl} - \pi_e \qquad (1)$$

where γ_{lv}, γ_{sv} and γ_{sl} are the free energies of the liquid and solid against their saturated vapour and of the interface between liquid and solid respectively and π e is the equilibrium pressure on the solid. θ is the angle of contact between a liquid droplet and a plane solid surface. When $\theta = 0$, the liquid is considered to completely wet the solid and when $\theta \neq 0$, the surface is less wettable. The wettability of a material is favoured by low interfacial free energy, high solid surface free energy and low liquid - surface free energy [204]. Further modification of equation (1) allowed Owens and Wendt to calculate the value of θ as follows:-

$$\text{Cos. } \theta + 1 = 2/\gamma \; [(\gamma^{d} \gamma^{d}_{s})^{1/2} + (\gamma^{p}_{l} + \gamma^{p}_{s}) 1/2] \qquad (2)$$

where γ^{d}_{l} is the dispersive component of a liquid,

γ^{d}_{s} is the dispersive component of the solid,

γ^{p}_{l} is the polar component of the liquid,

γ^{p}_{s} is the polar component of the solid.

The value of the contact angle is affected by the atoms that are exposed in the upper 10 Å of the material surface. Thus, in this study, contact angles were measured by the sessile drop method in air, as described by Owens and Wendt [204]. However, prior to analysis by this technique, all the hard and soft contact lenses were washed in PBS, rinsed thoroughly in distilled water and air dried. Drops of the wetting liquids (water and methylene iodide) were then placed onto separate areas of the substrate. Each drop was then viewed through a rotating eyepiece calibrated in degrees, and the contact angle, θ, at each side of the side of the drop was measured. From the mean values of θ, measured using both water and methylene iodide, the polar and dispersive components were calculated using the equation (2).

2.12. Extraction procedures.

2.12.1. Foetal calf serum lipid extraction.

To 25ml of foetal calf serum (FCS) 100ml of chloroform/methanol mixture (2:1v/v) were added. The two layers were separated using a separating funnel, and the upper non-lipid containing layer discarded. The lipid containing fraction was filtered (Whatman filter paper 541) and the filtrant washed through with chloroform/methanol mixture (2:1 v/v). The filtrate was washed with 100ml of 0.88M KCL (Folch wash) x 3 [205,206], the upper layer being discarded after each application. The remaining lipid fraction was then dried down under nitrogen and frozen prior to use. Controls were also carried out where the procedure was undertaken in the absence of serum.

2.12.2. Extraction of adsorbed material on contact lenses.

As a result of assessment procedures described in chapter 3 the following procedure was adopted. To a single spoilt contact lens, 3ml of methanol was added. The vial was covered with tin foil, topped with Nescofilm to prevent extraction from the Nescofilm and aerial contamination, and shaken for 30 minutes. This methanol extract is then dried under nitrogen and frozen prior to use. The lens was then rinsed and stored overnight in preserved saline. After storage 3ml of 1% SDS was added to the contact lens and placed on a shaker for 30 minutes. The SDS solution is then stored between 0-5°C prior to use. The lens is then stored in preserved saline. The remaining material on the lens surface may also be treated with a commercial surfactant or enzyme cleaner, if required. These extracts are then analysed by a number of techniques.

2.13. Thin layer chromatography.

Analysis of both foetal calf serum (FCS) and the adsorbed lipid layer extracts were carried out in duplicate. Using this technique the adsorbent was held on a glass plate as a thin layer. Silica gel plates (Anachem Merc.) measuring 20cm x 5cm were heat activated at 80°C for 1 hour, after which time a 2mm margin was scraped off either side of the plate. Standards and samples, dissolved in chloroform, were applied as discrete spots 1.5-2cm over the bottom of the plate.

2.13.1. Procedure for thin layer chromatography.

The following solvent mixture and standards were typically used:

Solvent (v/v):	Hexane	Diethyl ether	Acetic acid.
	70	30	2

Standards: Dipalmitin

Fatty acids (linolenic, oleic, linoleic, arachidic, stearic, palmitic)

Phospholipid (Phosphatidyl-L-serine, phosphatidylcholine)

Triglyceride (trilaurin, trilinolein)

Cholesterol ester (palmitate, oleate, linoleate)

Monostearin.

Lanolin

Cetyl alcohol

Cholesterol.

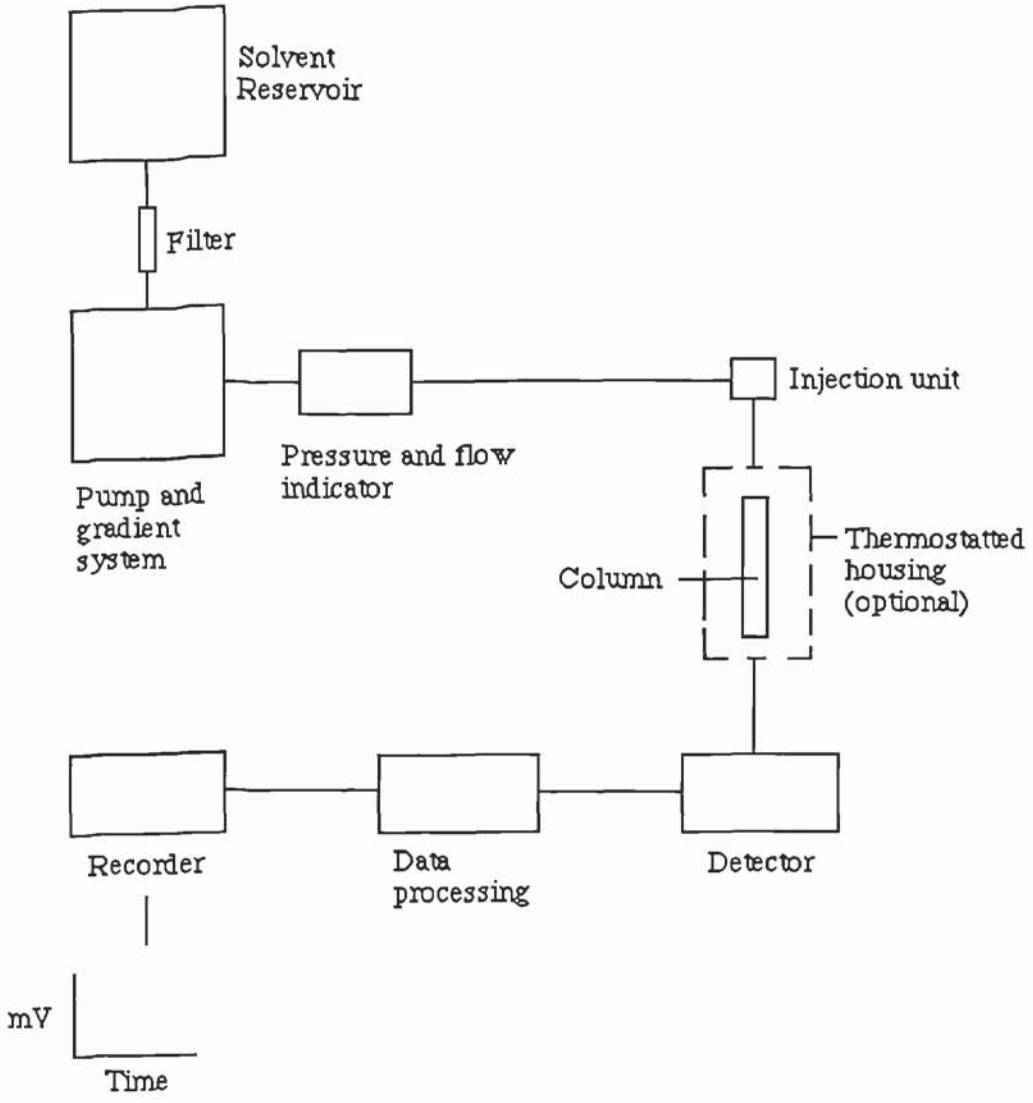
The plates were placed into the solvent mixture and allowed to run until the front was 5cm from the top of the plate (approximately 0.5 hour). Both plates were then air dried and sprayed with 0.1% bromothymol blue in 10% ethanol. The circumference of each spot was then outlined by indentations into the silica gel [207].

2.14:- High Performance liquid chromatography (HPLC).

High performance liquid chromatography (HPLC) is a method of separating a mixture of solutes. The sample mixture is injected onto a column of narrow bore containing a porous partitioning material (stationary phase). The mobile phase (solvent) is pumped down the column under pressure. This elutes the injected sample. Under the correct conditions, different components of the injected sample will migrate down the column at different rates. As a result discrete bands are formed and separate out as the migration proceeds. These bands can then be detected as they pass out of the column by for example, ultra-violet (UV), fluorescence, flame ionisation detectors [208-210] etc.. A

typical HPLC set up is shown in figure 2.3.

Figure 2.3:- A typical high performance liquid chromatography (HPLC) set up.



2.15. Electrophoresis.

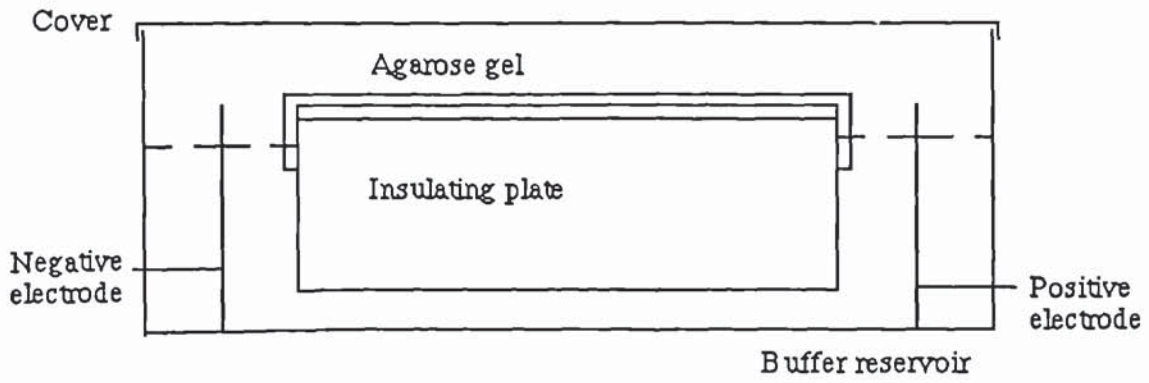
Electrophoresis is the transport of particles through a solvent by an electric field. The charged particles migrate towards the electrode with a charge opposite to its own. The

particle movement being dependent on the charge, size and shape of the particle, the strength of the applied electric field, the buffer and the composition of the supporting medium [211].

This technique can therefore, be used to characterise macromolecules by their rate of movement in the electric field e.g. protein molecular weight, distinguish molecules by net charge etc..

The separation of proteins in the SDS extracts from contact lenses was achieved with the use of a Beckman Paragon Serum System using a sensitive silver staining technique to identify them [212].

Figure 2.4:- Electrophoresis system.



CHAPTER 3.

A preliminary experimental examination of *in vivo* spoilation.

3.1. Introduction.

This chapter describes a preliminary experimental examination of *'ex vivo'* spoiled ocular lenses. The work was carried out to assess the manifestations of these phenomena and to enable the applicability of a few analytical techniques to be examined. Essentially, these studies provided the basis upon which decisions relating to the choice of analytical techniques and experimental procedure for further development were made. It is important to note that they represent typical results and simply form the basis for the development of the more specialised analytical studies in chapters 4-8.

Ocular spoilation results from the interaction of the contact lens with the microenvironment. As previously stated the chemical composition of the lens deposits is extremely complex and nearly all the chemical components found to be present in the deposit are apparently derived from tear film. The major problem, especially in the long term, which results from biological interfacial conversion is the discrete elevated deposit or 'white spot'. Originally these deposits were suggested to be simple inorganic or proteinaceous deposits. The structures are morphologically and chemically complex. They are now known to contain high proportions of lipids and are extremely resistant to both physical and chemical removal.

The role of the lipids in the initiation of the formation of these white spots and in a common biological interfacial conversion process is presently unclear. Where ever white spots are formed they have a very similar morphology and composition. Additionally changes in surface chemistry of the lens matrix affects the rate of white spot formation, rather than the ultimate fate of the lens. By changing the wear protocol, method of

sterilization and composition of the storage solutions the path of spoilation can be completely changed. In such cases, the white spot formation process still applies, but its importance can be eclipsed by the more rapid onset of other types of the spoilation process. For example, enhanced lysozyme deposition occurs at surfaces carrying a high negative charge, formation of melanin within the lens matrix and precipitation of inorganic films where the solubility products of the ions are exceeded.

Thus, the study of the discrete elevated deposit is difficult, due to its complex nature, rapid formation and the minute quantities of deposited material involved. These preliminary studies involved the observation and analysis of the formed deposits.

3.2. Optical microscopy.

The contact lenses were stored in preserved saline after being cleaned using a cold regime to stop microbacterial contamination. The lenses were then only handled with clean forceps. The contact lenses were then mounted for optical microscope examination on clean glass slides and flattened using a cover slip.

The complex multi-layered lobular morphology of the discrete elevated or white spot deposit [140,141] is clearly observable under the optical microscope (figures 3.1, 3.2). Some deposits appear less lobular than others and their shape varies considerably (figures 3.3, 3.4, 3.5, 3.6). Some deposits appear to be covered by a film, possibly protein and particulate material (figures 3.7, 3.8). Groups of white spots were also regularly seen (figure 3.9). Other deposits are linked or surrounded by films (figures 3.10, 3.11, 3.12). The appearance of these films is variable (figures 3.13, 3.14, 3.15) and it is

difficult to distinguish between them by visual examination only. The inorganic films tend to be more crystalline in appearance, but are often covered by protein films.

Fluorescence microscopy showed distinctive autofluorescence patterns were obtained with discrete elevated deposits (figures 3.16, 3.17). The lobulated components situated near the base of the deposit appear to be the primary autofluorescent sites.

The sections through the deposits showed that although the bulk of deposit occurs polymerised on the lens surface, some penetration into the matrix is however achieved (figures 3.18, 3.19, 3.20). The progressive involvement of the growing deposit with the lens surface is shown in figure 3.19 .

Figure 3.1:- Discrete elevated deposit, Permaflex X125.

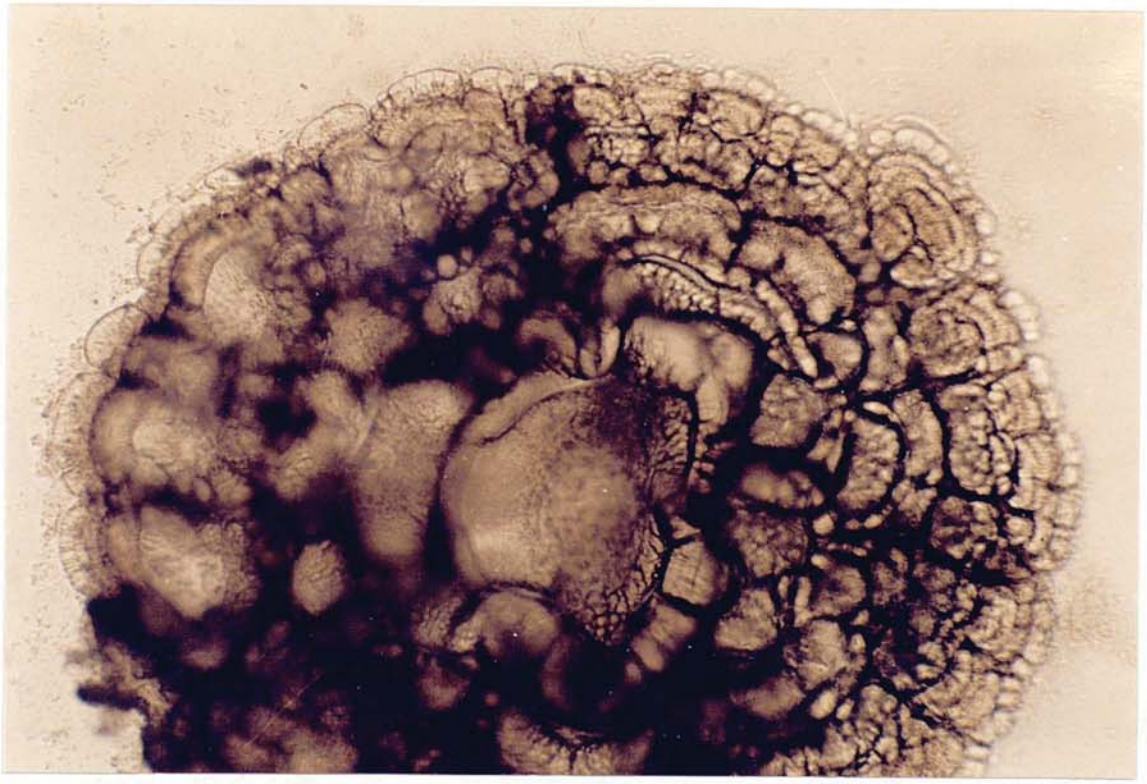


Figure 3.2:- Discrete elevated deposit, Permaflex X125.

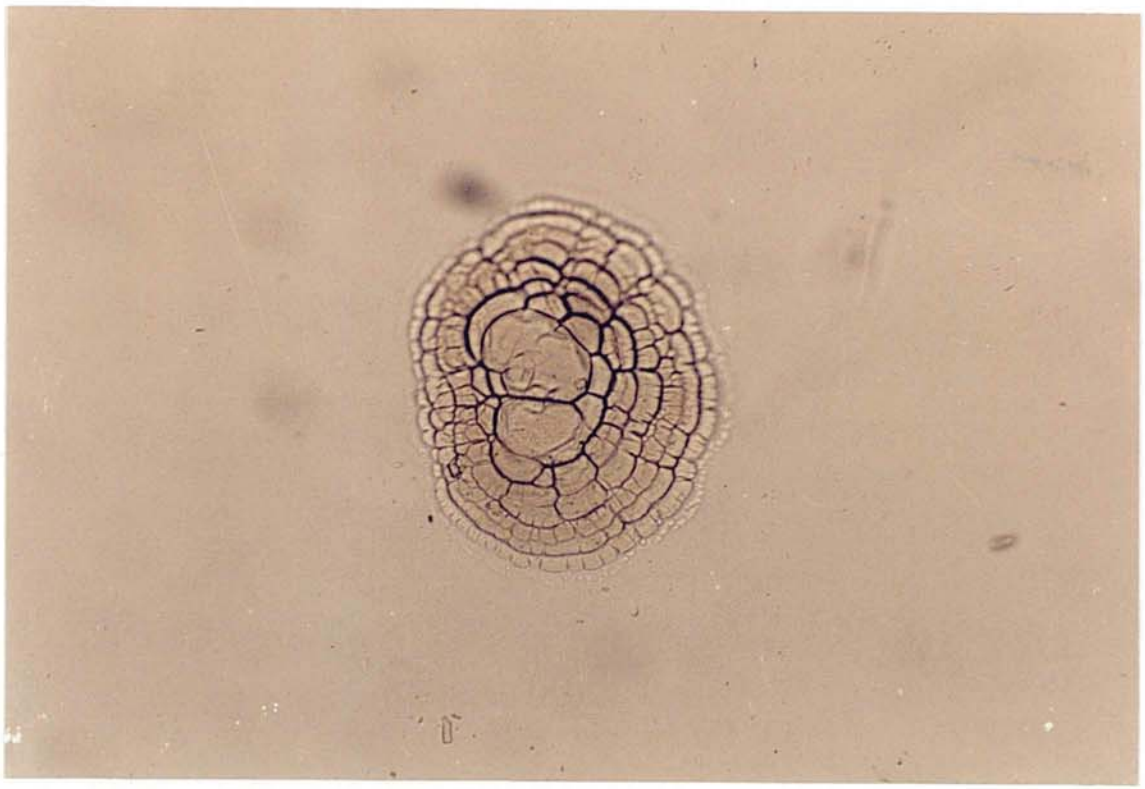


Figure 3.3:- Discrete elevated deposit, X-ten X125.

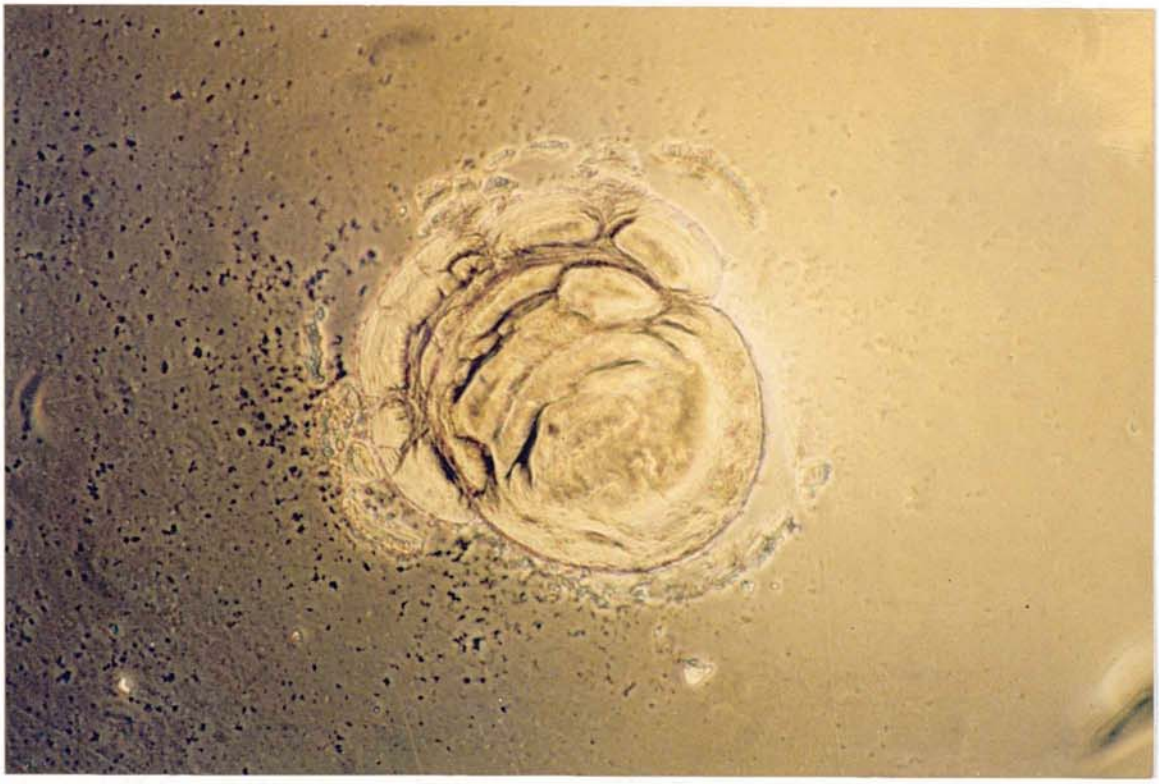


Figure 3.4:- Discrete elevated deposit, X-ten. Seen under phase contrast, X125.

Figure 3.5:- Discrete elevated deposit, X-ten. Seen under phase contrast, X125.

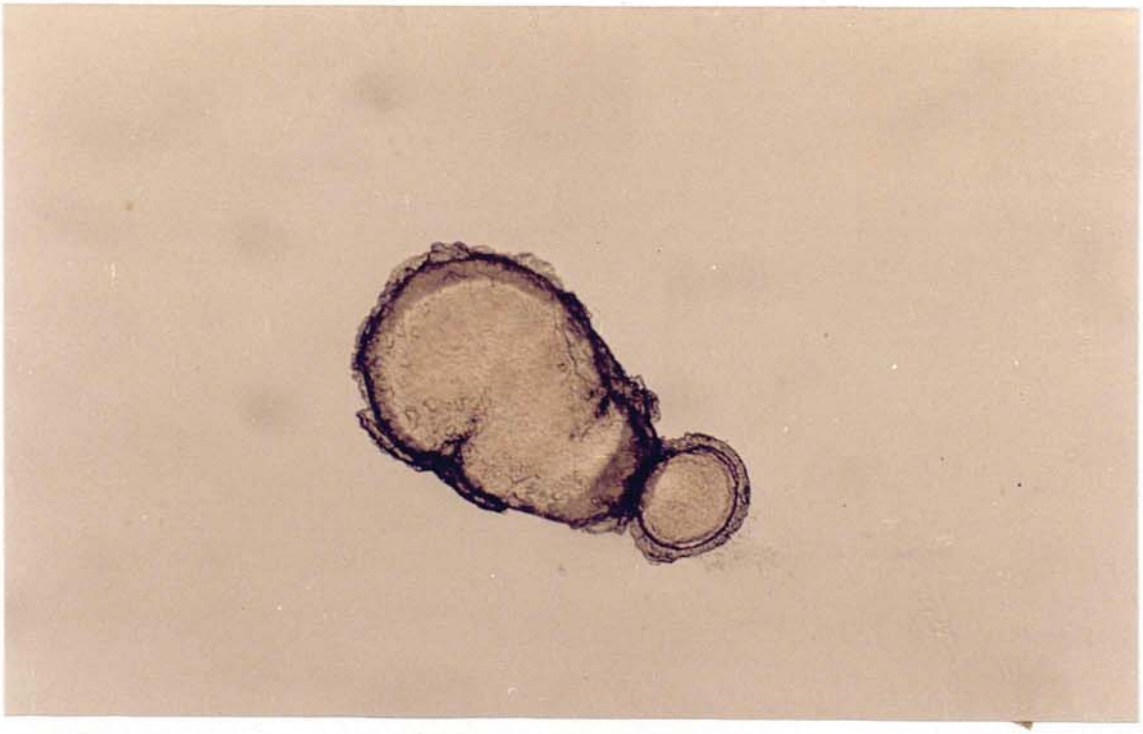


Figure 3.6:- Discrete elevated deposit, Permaflex X125

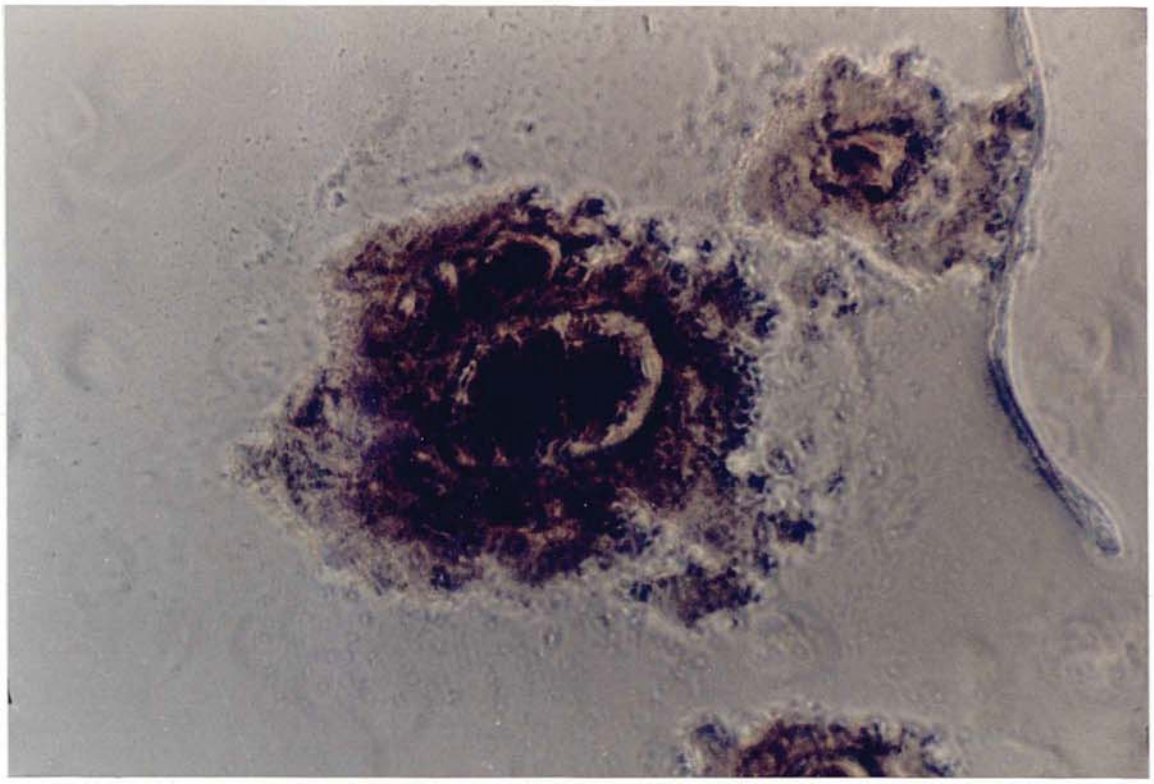


Figure 3.7:- Discrete elevated deposit, Permaflex X125.

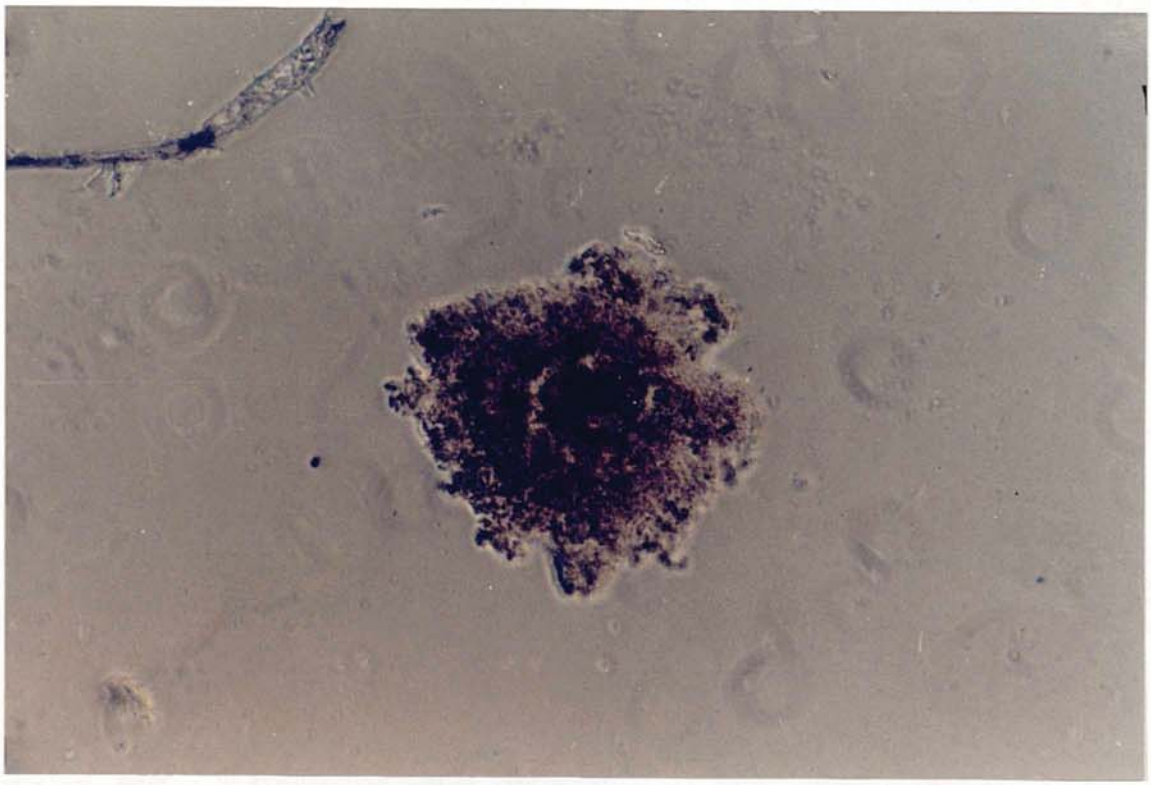


Figure 3.8:- Discrete elevated deposit, Permaflex X125.

Figure 3.9:- A concentrated group of discrete elevated deposits, X-ten X125.

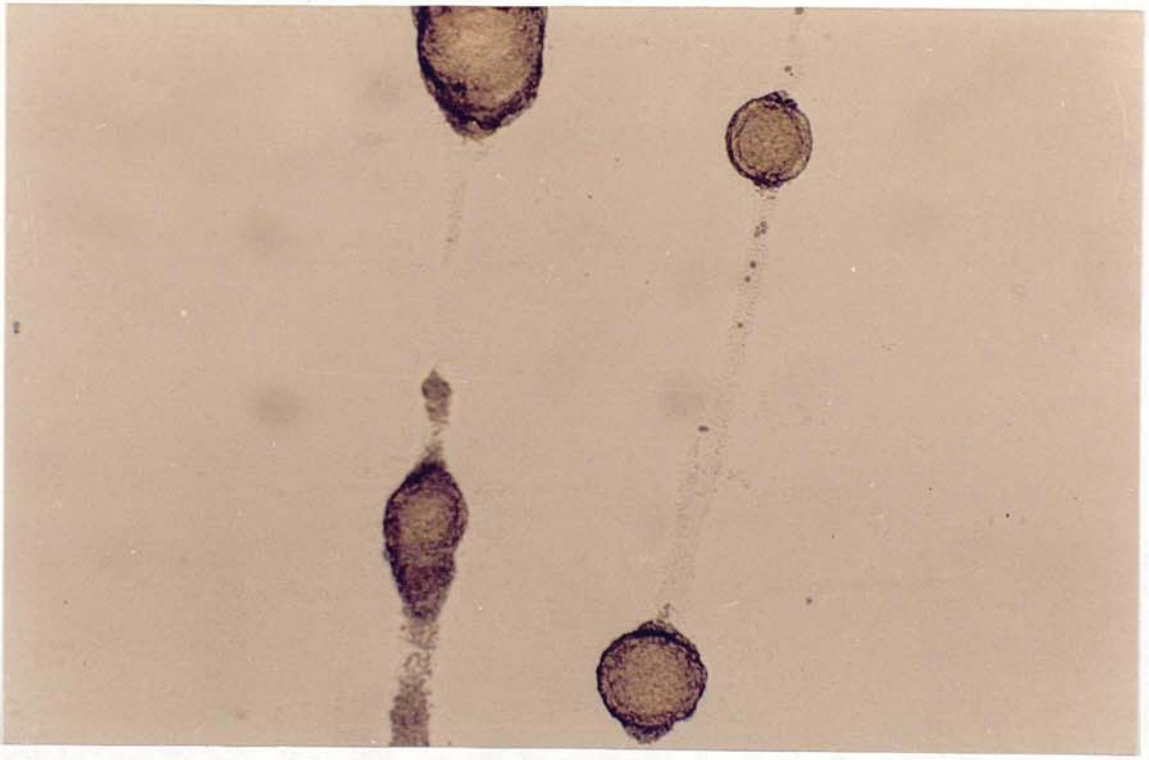


Figure 3.10:- Discrete elevated deposits linked by a film, Permaflex X125.

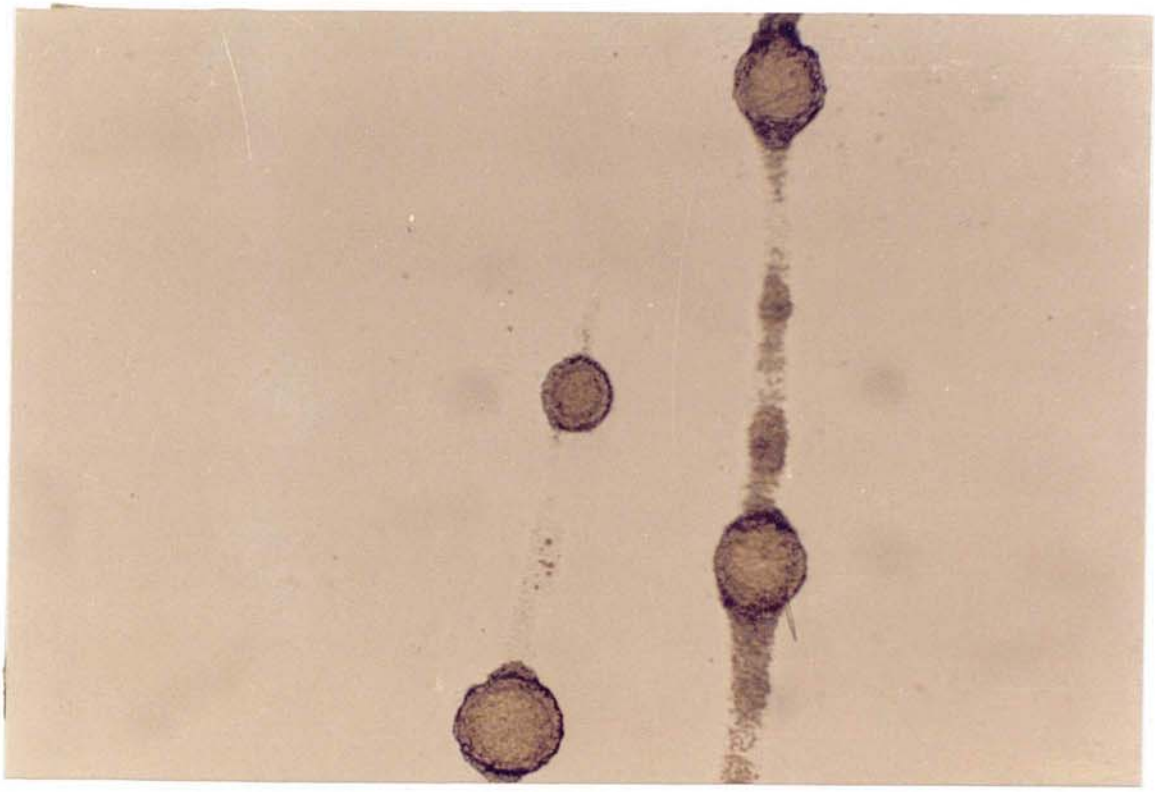


Figure 3.11:- Discrete elevated deposits showing the film build-up, Permaflex X125.

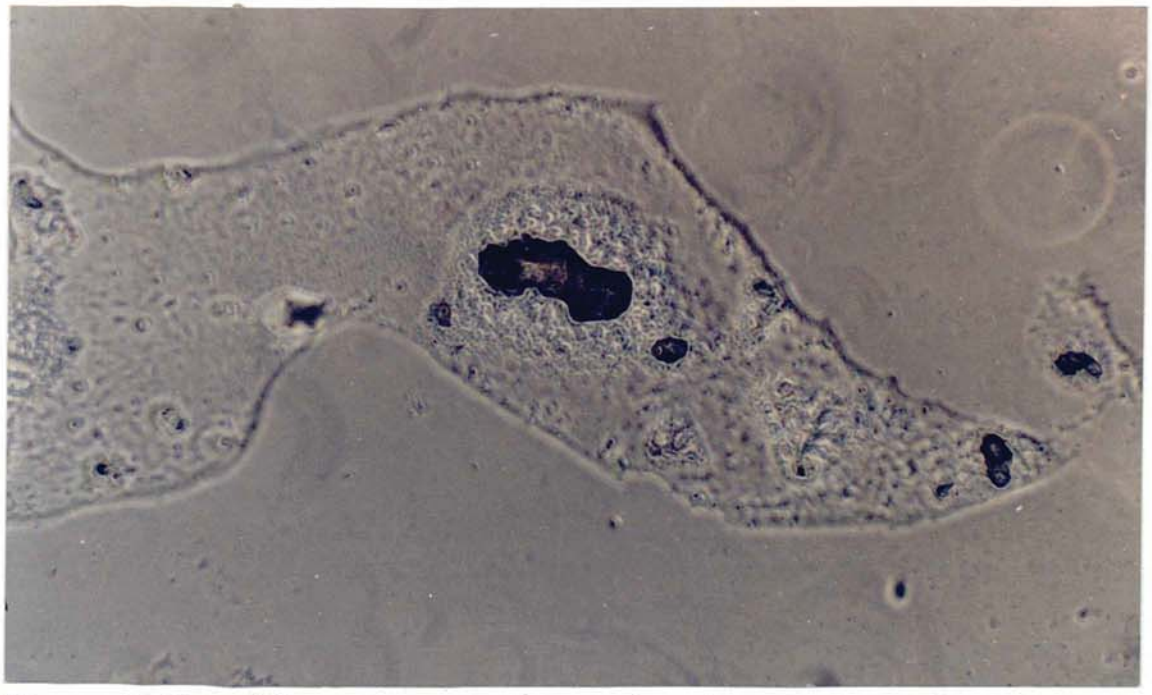


Figure 3.12:- Discrete elevated deposits situated in a film, Permaflex X125.

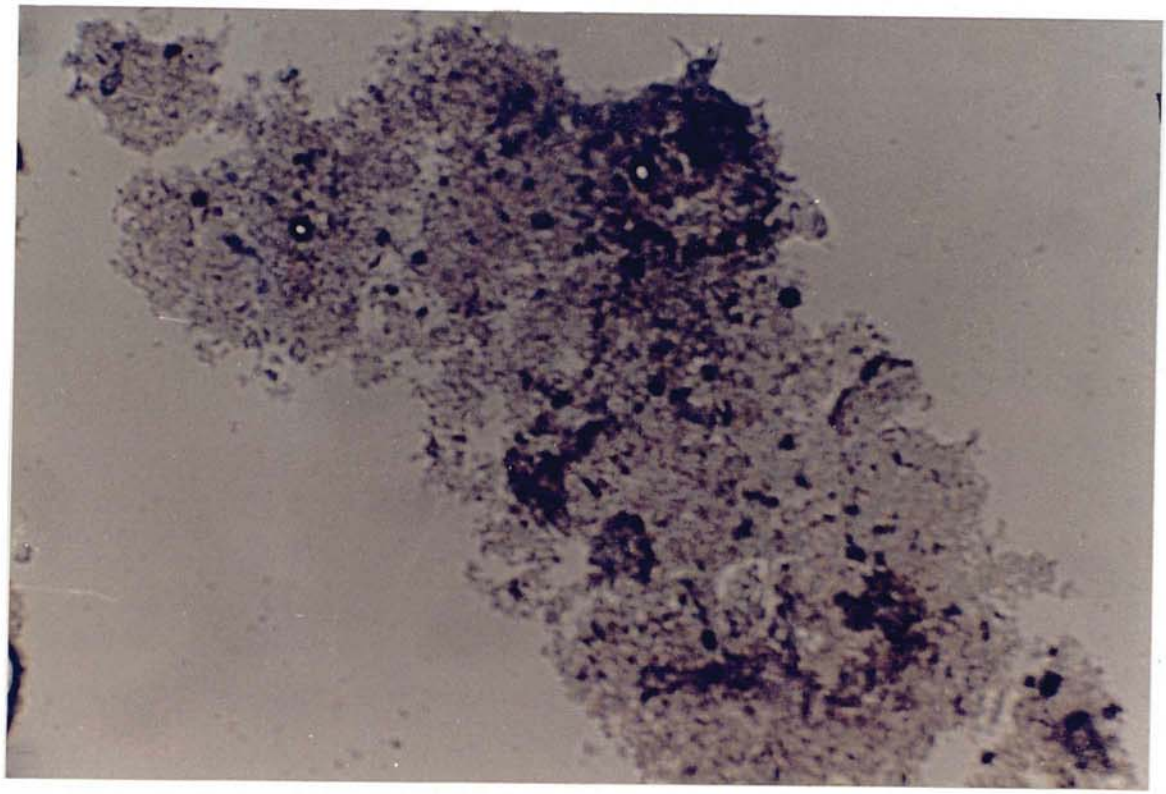


Figure 3.13:- Surface film on a Permaflex lens, X125.

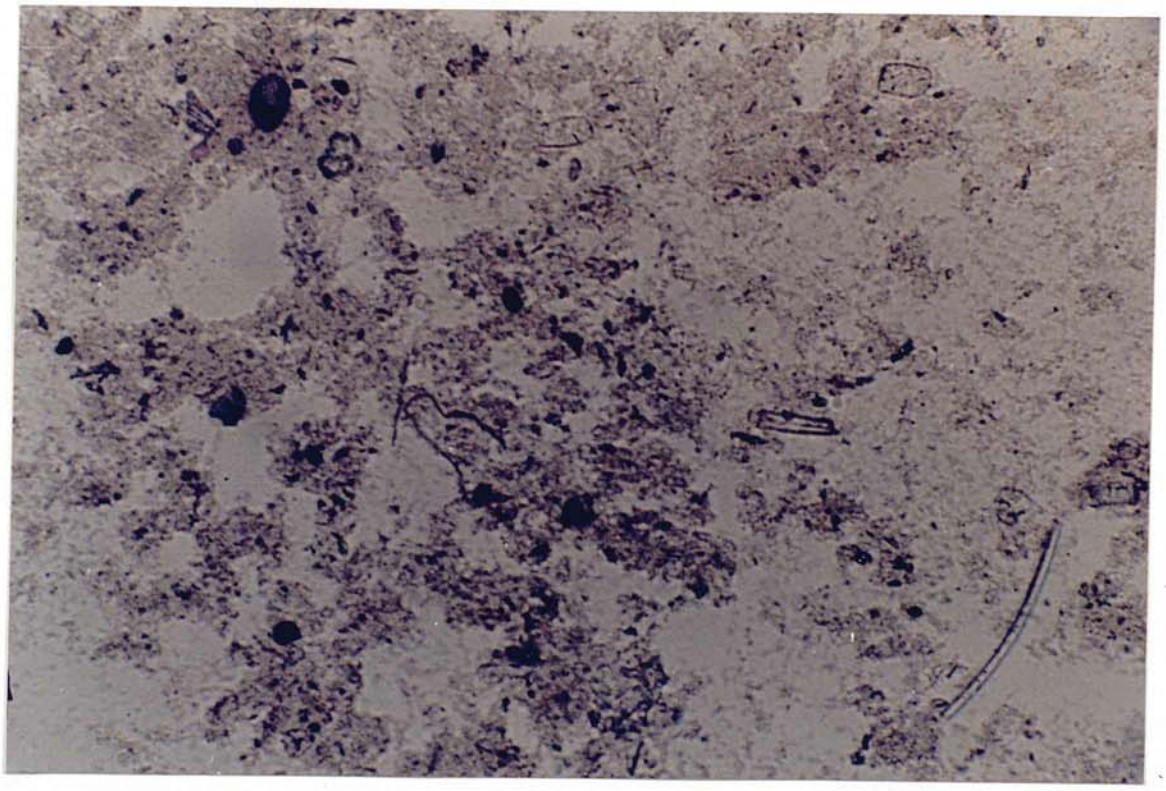


Figure 3.14:- Surface film on a Permaflex lens, X313.

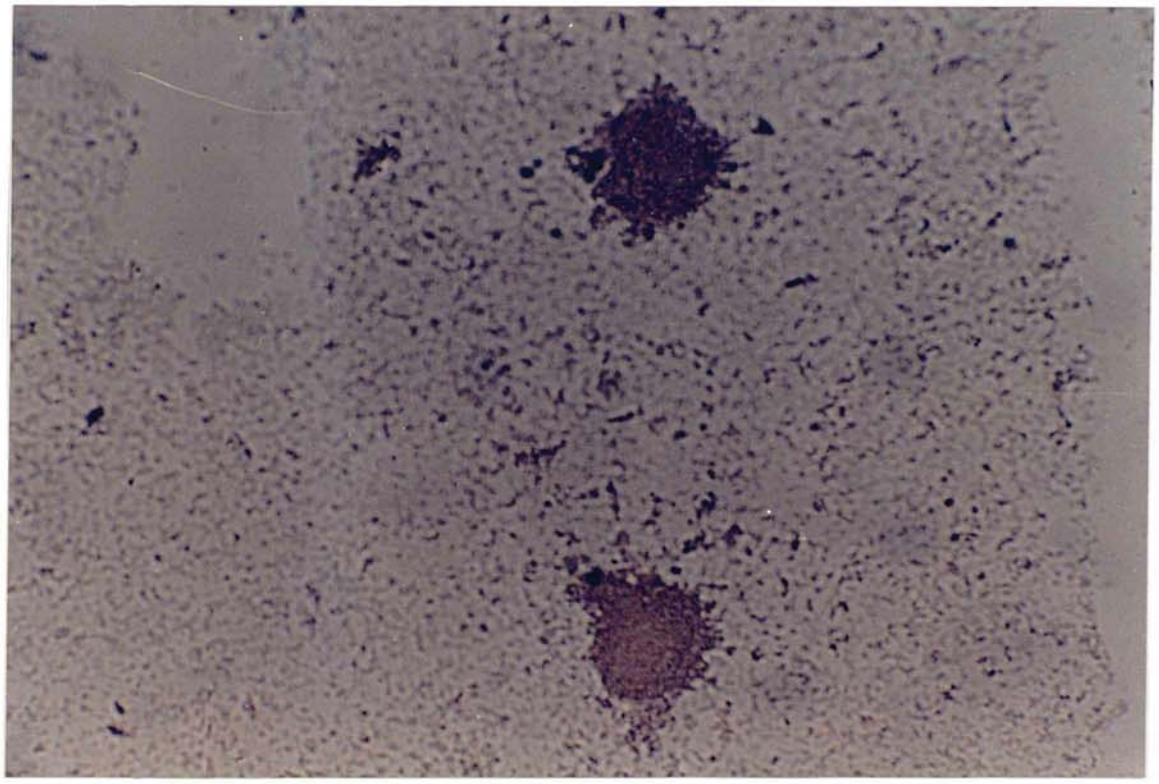


Figure 3.15:- Surface film on a Permaflex lens, X125.

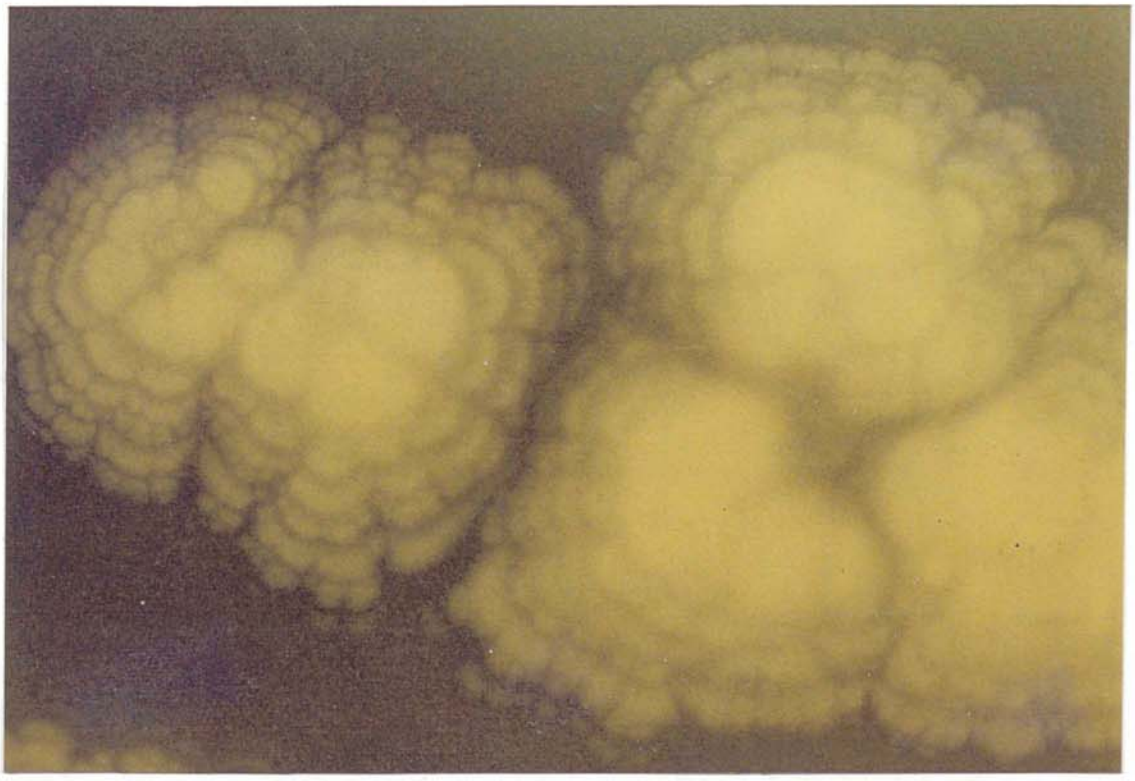


Figure 3.16:- Autofluorescent profile of a discrete elevated deposit, Permaflex X125.

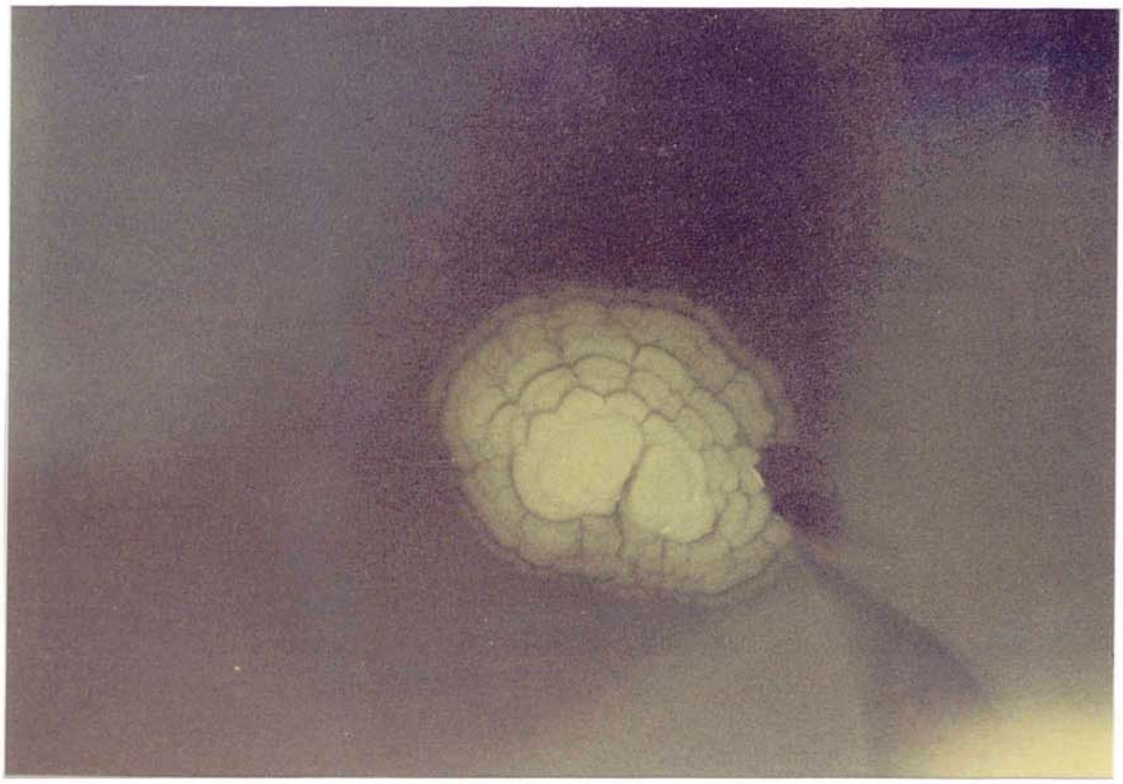


Figure 3.17:- Autofluorescent profile of a discrete elevated deposit, X-ten X125.

Figure 3.18:- L.S. of a discrete elevated deposit, Permaflex X125.

Figure 3.19:- L.S. of a discrete elevated deposit, Permaflex X313.

Figure 3.20:- L.S. of a discrete elevated deposit, X-ten X125.

3.2. Scanning electron microscopy.

Electron micrographs of discrete elevated white spot deposits prepared using conventional biological fixation techniques produced results of the form shown if figure 3.21. The use of buffered gluteraldehyde-picric acid for 90 minutes at 4°C, proved beneficial in causing minimal disruption and dissolution of the interfacial deposit layers. Picric acid was employed due to its improved lipid fixation [213]. The presence of the primary plateau upon which the 'white spot' deposit is subsequently formed is visible using this fixative (figure 3.22). The initial primary layer can also be observed using lenses worn for short periods (figure 3.24). These primary layers are covered by the growing deposit as it spreads, although the plateau region is clearly visible around the periphery of deposits.

Figure 3.21:- Electron micrograph of a discrete elevated deposit prepared using conventional biological fixation.

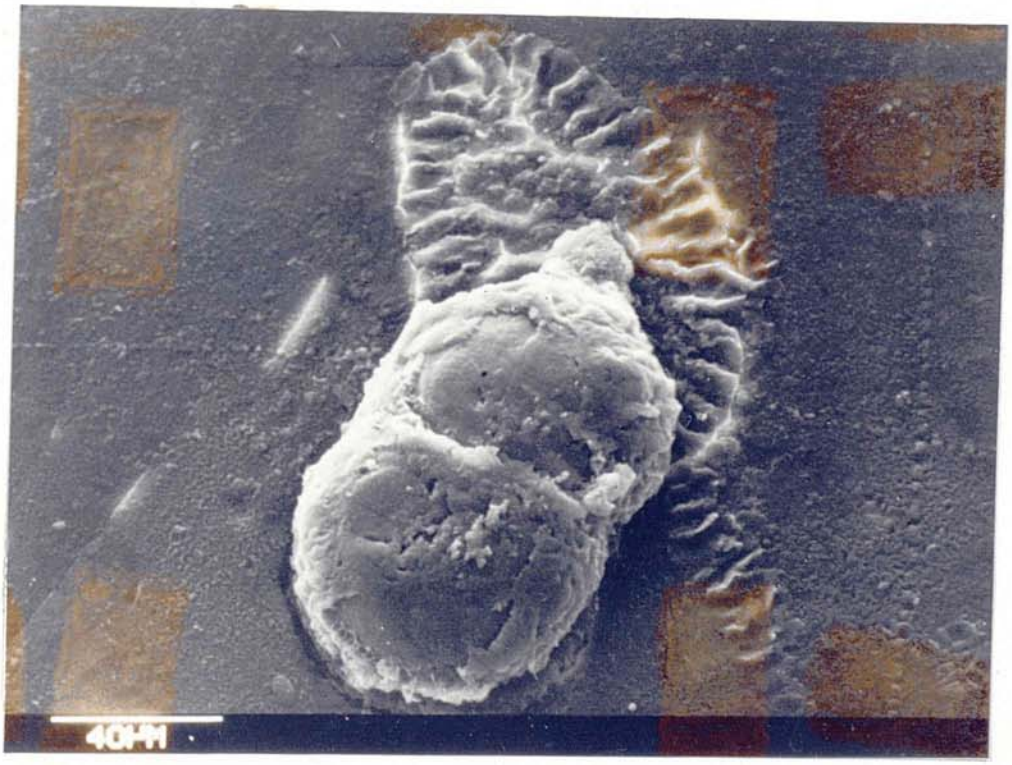


Figure 3.22:- Electron micrograph of a discrete elevated deposit prepared using picric acid fixation.

Figure 3.23:- Electron micrograph of a discrete elevated deposit showing the segmented morphology.

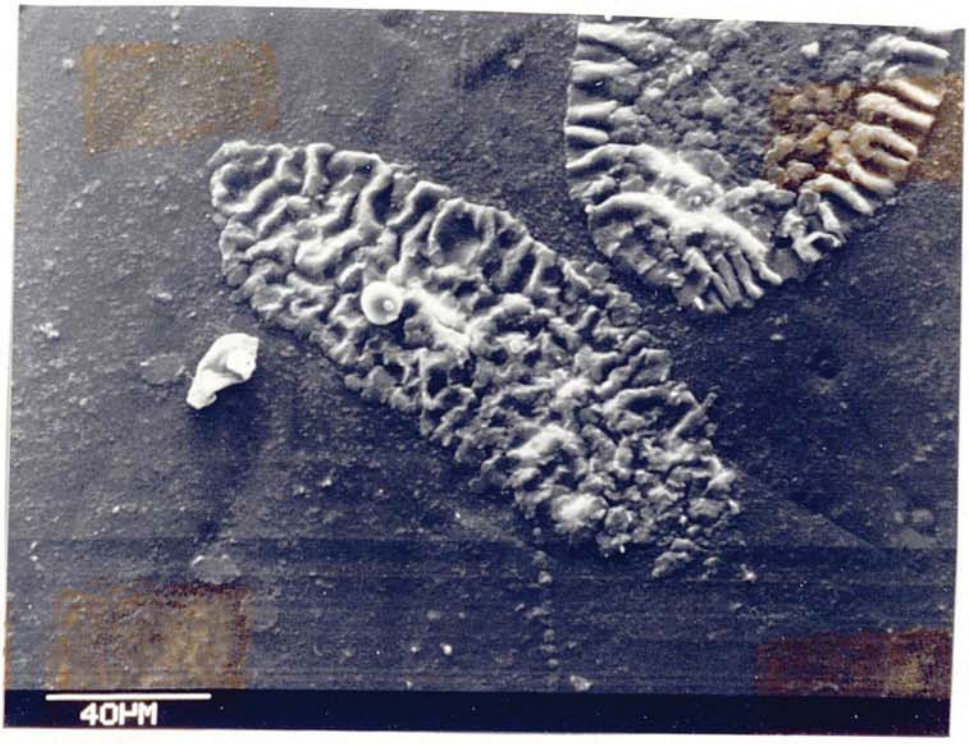


Figure 3.24:- Electron micrograph of the primary layer of a discrete elevated deposit.

3.3. Histological staining.

The lenses were fixed in 10% buffered formaldehyde (pH 7.4) for 12 hours at room temperature. The stains used to study the deposit morphology are summarised in table 3.1.

The stain formulations employed were laid down by Pearse [196]. In all cases normal dehydration procedures were eliminated since it was found that such steps minimized shrinkage and distortion caused by this process.

Table 3.1:- Stains employed in the detection of deposit components.

Stain	Selectivity
Oil Red O	Lipids
Digitonin	Cholesterol
Performic Acid Schiff (PFAS)	Unsaturated lipids
Bromine silver nitrate	Unsaturated lipids
Periodic Acid Schiff (PAS)	Carbohydrates
Mucicarmine	Mucins
Millon's reagent	Proteins
Von Kossa	Calcium

Deposit sections stained positively with Oil Red O in a consistent and uniform

fashion throughout the deposit (figure 3.25). This staining is limited to the deposit material and does not stain the hydrogel matrix. Positive staining was also obtained with digitonin (figure 3.26), the staining being most intense at the deposit/matrix interface, and PFAS (figure 3.27). The penetration of the deposit into the lens matrix and the progressive involvement of the lipoidal species throughout the deposit can be clearly seen in figure 3.28. This shows the positive reaction to bromine/silver nitrate, which reacts with unsaturated lipids, throughout the deposit structure. The lipid distribution throughout the deposit is not uniform. The deposits showed little staining with PAS and Mucicarmine. The deposit staining with Millon's reagent and Von Kossa stain was variable (figure 3.29).

Figure 3.25:- L.S. (Oil Red O) of a discrete elevated deposit. Phase contrast X260.

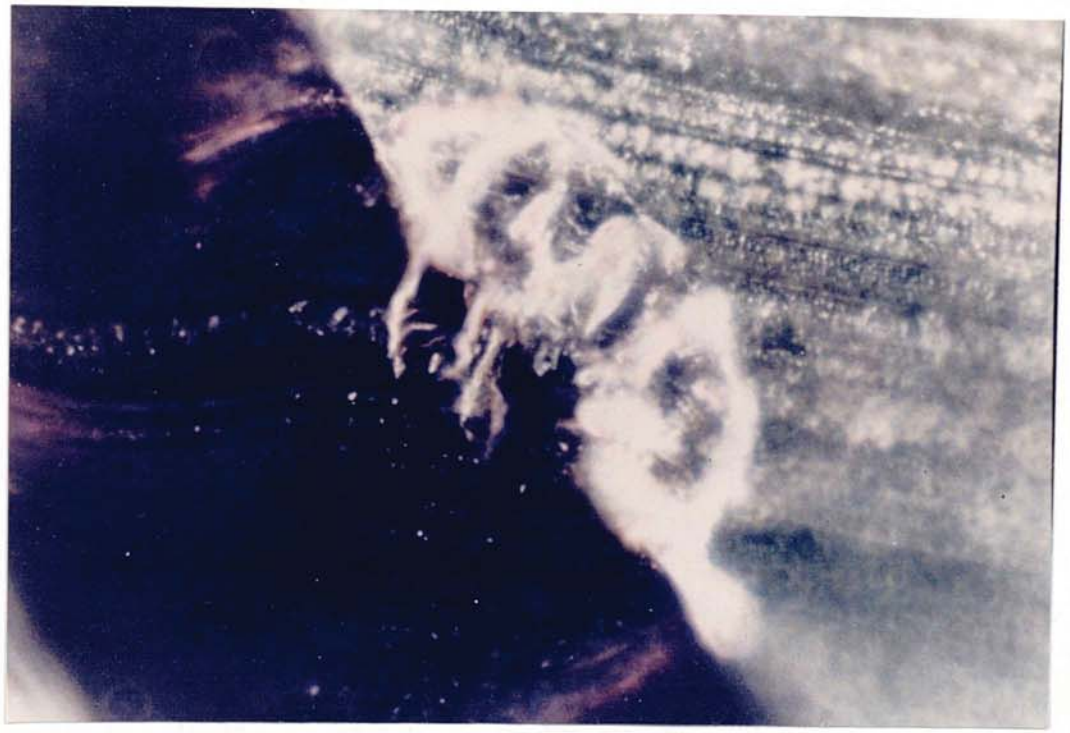


Figure 3.26:- L.S. (Digitonin) of a discrete elevated deposit. Phase contrast X260.

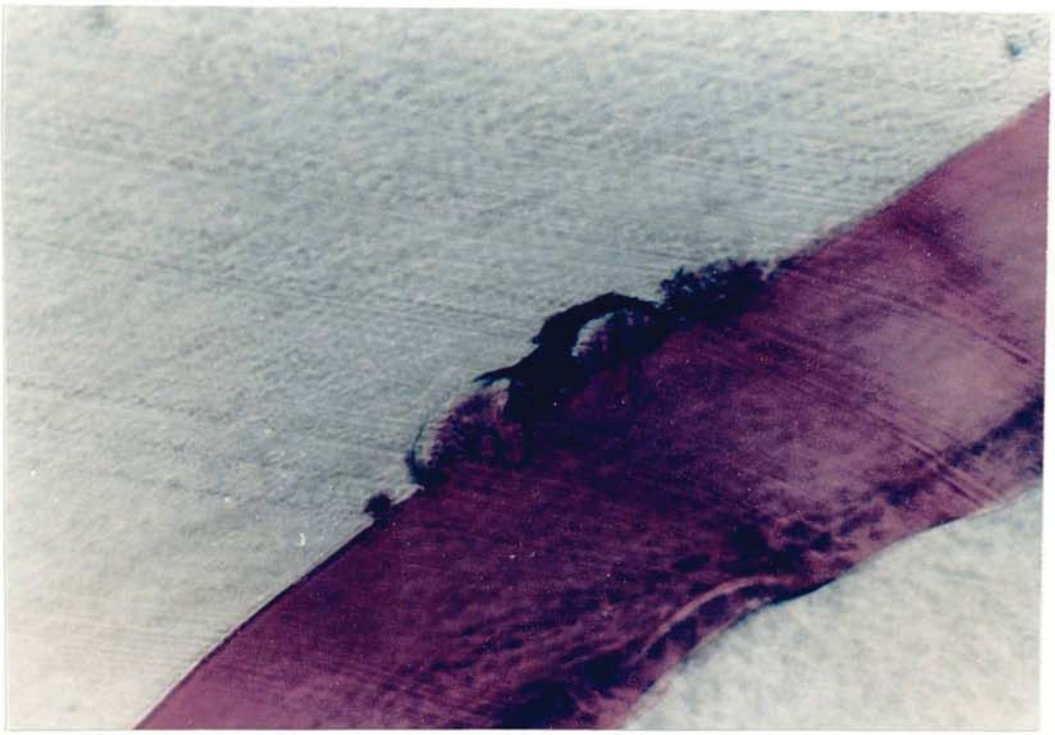


Figure 3.27:- L.S. (PFAS) of a discrete elevated deposit. Phase contrast X260.

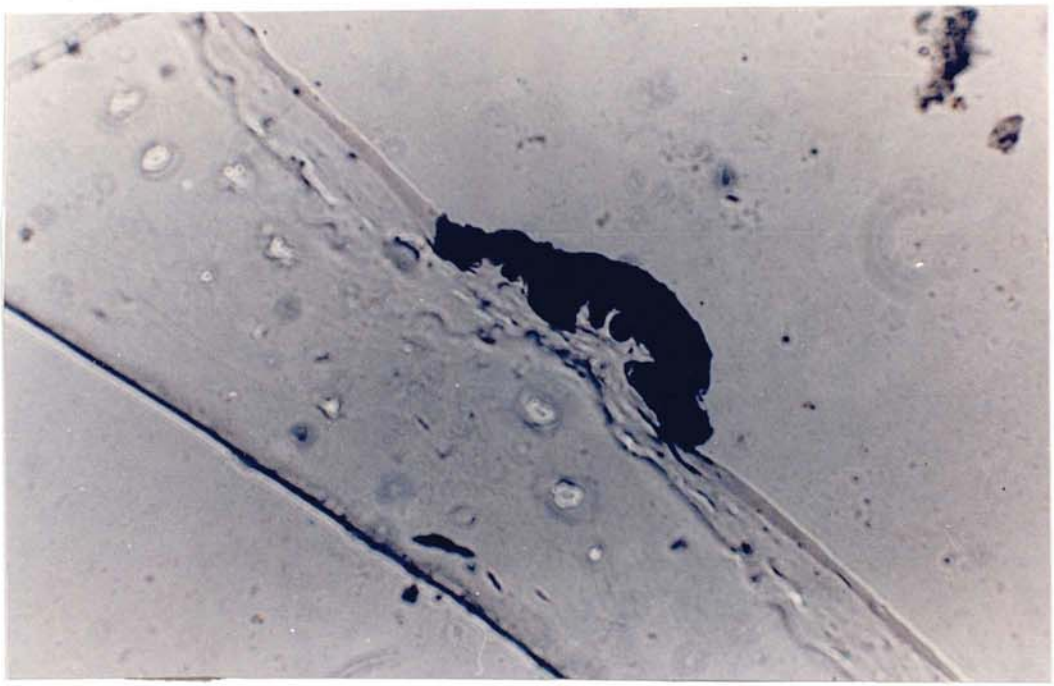


Figure 3.28:- L.S. (Silver nitrate) of a discrete elevated deposit. Phase contrast X260.

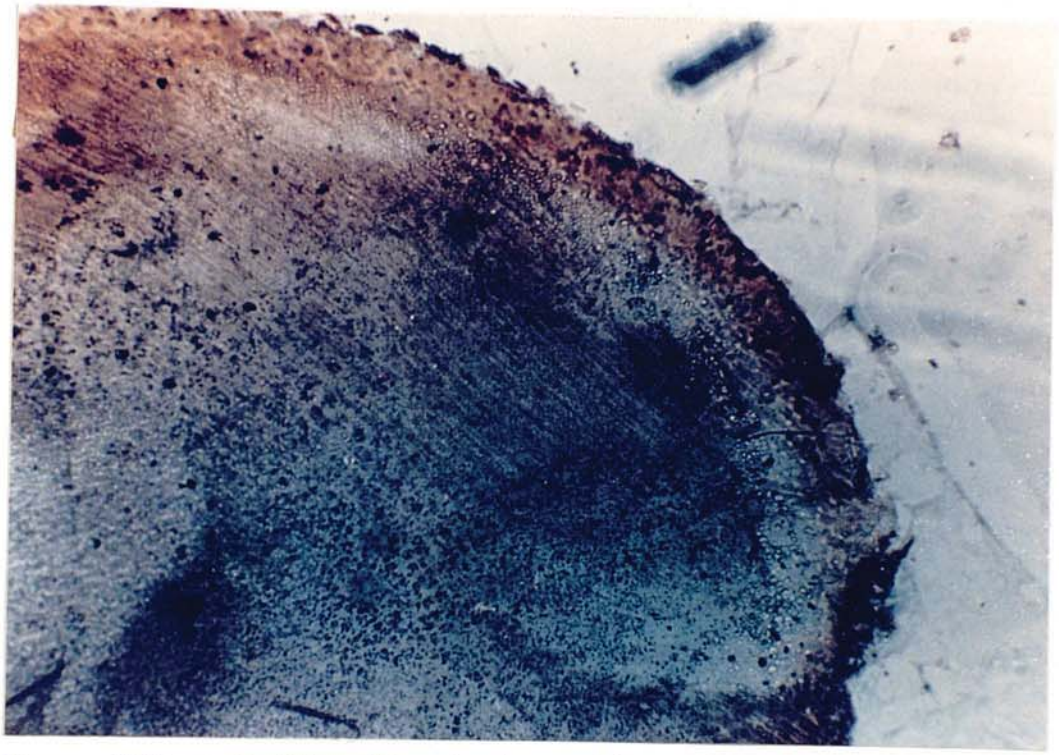


Figure 3.29: L.S. (Von Kossa) of a calcium film. Phase contrast X260

3.4. Thin layer chromatography.

Following the extraction of lipids from a single contact lens, using 3ml of methanol, the solvent was dried down under nitrogen and the sample frozen. Prior to analysis by thin layer chromatography (TLC) 200µl of chloroform were added to the sample vial and 50µl of this applied to the activated TLC plates as small spots. In addition to the samples, known standards were also applied to the plates. Each plate was placed into the solvent mixture (Hexane, diethyl ether, acetic acid 70:30:2ml) for a period of 20-30 minutes, after which time the plates were removed air dried and sprayed with 0.1% bromothymol blue in 10% ethanol. After developing the R_f values for each of the sample spots and standards were determined.

Table 3.2:- R_f values for lipid standards used in TLC analysis of a single soft contact lens.

Lipid	Distance moved	R_f value	R_f value range.
Phosphatidyl-L-serine	0.0-0.25	0.01	0.0-0.01
Phosphatidylcholine	0.0-0.25	0.01	0.0-0.01
Phosphatidylinositol	0.0-0.25	0.01	0.0-0.01
Monostearin	0.16-0.98	0.04	0.03-0.05
Cholesterol	1.54-2.0	0.13	0.13-0.14
Dipalmitin	1.6-2.4	0.19	0.16-0.21
Cetyl alcohol	2.4-3.9	0.21	0.2-0.22
Linoleic acid	3.6-4.8	0.31	0.30-0.34
Oleic acid	3.8-5.1	0.33	0.32-0.34
Linolenic acid	3.6-4.8	0.30	0.29-0.31
Arachidic acid	4.0-5.2	0.35	0.32-0.38
Stearic acid	3.6-5.2	0.33	0.31-0.34
Palmitic acid	3.6-5.4	0.33	0.33-0.34

Table 3.2:- (continued) R_f values for lipid standards used in TLC analysis of a single soft contact lens.

Lipid	Distance moved	R_f value	R_f value range.
Triolein	7.3-8.3	0.62	0.58-0.67
Trilinolein	6.4-7.8	0.55	0.54-0.57
Trilaurin	6.8-7.9	0.57	0.55-0.61
Cholesterol palmitate	10.3-11.8	0.86	0.84-0.87
Cholesterol oleate	10.4-11.3	0.83	0.70-0.90
Cholesterol linoleate	10.0-11.0	0.84	0.80-0.87

Table 3.3:- R_f values for extracted lipids from a single contact lens.

Lens	Distance moved	Rf value	Lipid
X-ten 58	1.5-2.0	0.14	Cholesterol
	3.2-3.7	0.28	Fatty acid
	6.2-8.2	0.59	Triglyceride
	10.2-11.5	0.89	Cholesterol ester
X-ten 70	1.3-2.0	0.14	Cholesterol
	2.7-4.0	0.27	Fatty acid
	6.0-7.9	0.57	Triglyceride
	10.3-11.1	0.89	Cholesterol ester
X-ten 307	1.3-1.8	0.13	Cholesterol
	2.8-3.8	0.27	Fatty acid
	6.1-8.0	0.57	Triglyceride
	10.4-11.4	0.88	Cholesterol ester
X-ten 308	1.3-1.8	0.13	Cholesterol
	3.1-4.2	0.29	Fatty acid
	6.1-8.1	0.57	Triglyceride
	10.5-11.5	0.89	Cholesterol ester.

The results indicate the presence of cholesterol, fatty acids, triglycerides and cholesterol esters. This is in agreement with previous work, which detected cholesterol,

cholesterol esters, triglycerides, monoglycerides, fatty acids and fatty alcohols [142-145]. However not all these lipid classes are always detected, dependent on the analytical technique used [146].

3.5. Electrophoresis.

There are several procedures for extracting protein from contact lenses including 6N Hydrochloric acid (HCL) [161]; Tris HCL buffer, 4% sodium dodecyl sulphate (SDS) and 10% B-mercaptoethanol [193]; heat SDS detergent and dithiothreitol (DTT) plus various chapotropic agents or ethylenediamine tetraacetic acid (EDTA) singly or in combination [167] and 1% SDS with or without warming [147]. Only lenses treated with 1% SDS and DTT were shown, by Welder, to have had the deposit completely removed. 1% SDS was shown by Bowers to be the most effective for protein extraction.

In order to test the effects of two proteins denaturing reagents; 1% SDS and 6M urea were utilised warm and cold. As a result of these tests 1% SDS was chosen to extract the proteins from the contact lenses.

The proteins were extracted from a single contact lens with white spot deposits, in 3ml of SDS on a shaker for 30 minutes. Prior to SDS extraction an extraction with 3ml of methanol was performed. This was tested for protein contamination by electrophoresis and the remainder used for TLC. Three standard protein solutions were also run; 0.1g/ml albumin, 0.07g/ml human-γ-globulins, 0.1/ml lysozyme. 3-5μl of each test sample and standard were applied to a Serum Protein (SPE) gel (Beckman). The gel was then run at 100V for 25 minutes. After electrophoresing the gel was silver stained according to the

100V for 25 minutes. After electrophoresing the gel was silver stained according to the method of Morrissey (1981) [212] with one alteration in that 0.1-0.5% sodium carbonate was used in the developer solution. After silver staining the gels were dried completely and photographed.

The effects of the methanol and SDS extraction on two lenses can be clearly observed (figures 3.30, 3.31). In both cases there is no trace of protein in the methanol tracts. Edge staining is probably due to protein contamination during handling. The SDS extracts appear to contain the proteins used as standards, albumin, lysozyme and globulins. These results relate to technique development and routine protein analysis would certainly now include other proteins for example lactoferrin as markers.

The results obtained show that albumin, globulins and lysozyme are present on the lenses. Whilst this is consistent with other workers [165-167] it is necessary to further analyse the proteins present in the deposits as other proteins are present in tears and some may also come from other factors e.g. enzyme cleaners, cosmetics, lens handling etc..

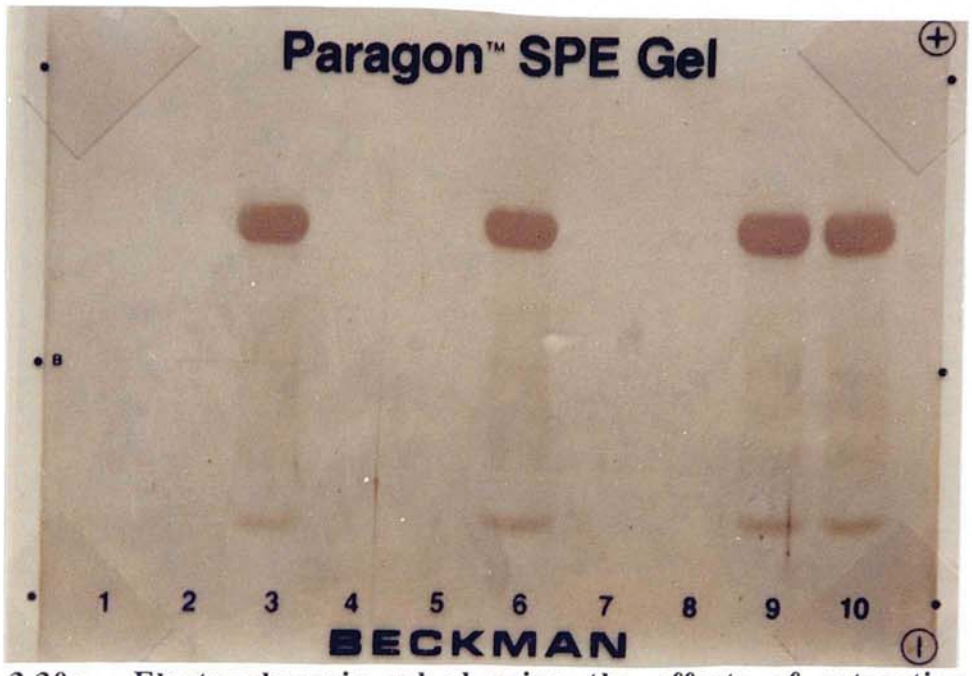


Figure 3.30:- Electrophoresis gel showing the effects of extraction on a Permaflex lens. Tracks 1, 2, 4, 5, 7, 8 are methanol extract; tracks 3, 6, 9 SDS extracts and track 10 in descending order a mixture of albumin, globulins and lysozyme.

Figure 3.31:- Electrophoresis gel showing the effects of extraction on an X-ten lens. Tracks 1, 2, 4, 5, 7, 8 are methanol extracts; 3, 6, 9 SDS extracts and track 10 in descending order a mixture of albumin, lysozyme and globulins.

3.6. Overview.

The manifestations of spoilation produced as a result of interfacial conversion exhibit many forms. The discrete elevated deposits display a complex multi-layered structure. These deposits are predominantly composed of tear-derived lipids laid down in a well-defined fashion. The primary layer is composed of unsaturated lipids, whilst the secondary and tertiary layers are dominated by cholesterol and cholesterol esters. A proportion of these lipids can be extracted for thin layer chromatography (TLC) analysis. The composition and location of deposition is unaffected by variations in wear protocol, chemistry of the lens matrix or tear composition of the lens wearers.

The presence of the variable protein component of these deposits is indicated by their reaction to Millon's reagent and the components extracted by SDS and resolved by electrophoresis.

The deposit reaction to PAS and Mucicarmine was insignificant. Whilst the deposit reaction to Von Kossa stain was variable, indicating that calcium is optional component in these deposits.

These results indicate the potential importance of the role of lipids in ocular spoilation. There is therefore a need to analyse the initial stages of the interfacial conversion process immediately upon insertion into the eye and after short time intervals in order to assess the extent and nature of numbers of lipoidal species. Extreme sensitivity of the analytical techniques which were developed to analyse the minute quantities of material is also a necessary requirement. Two main possible techniques are available for

exploitation; these are fluorescence spectroscopy and high performance liquid chromatography (HPLC). Fluorescence spectroscopy is a highly sensitive technique which may have a part to play in these studies. Similarly high performance liquid chromatography (HPLC) is a method frequently used in separating and detecting a mixture of biological components contained in a single solution. It too may be applicable to these optical phenomena. At the outset of this work, however neither technique had been successfully used to monitor the early stages of contact lens spoilation in either *in vivo* or *in vitro* studies.

CHAPTER 4.

Development of an High performance liquid chromatographic and fluorescence spectroscopy techniques to study ocular compatibility.

4.1. Introduction.

Microscopy and histological studies have shown that complex contact lens deposits include some of the components found in tears (e.g. proteins, amino acids, mucin, glycoproteins, glucose and lipids)[85,101]. The lipid class has been shown to consist of cholesterol, cholesterol esters, monoglycerides, diglycerides, triglycerides, fatty sterols, fatty alcohols and free fatty acids.

The first problem to be addressed in following these preliminary observations is that of development of an analytical technique which might enable lipoidal deposition on individual contact lenses to be investigated.

The involvement of the lipoidal species in ocular spoilation phenomena was demonstrated in the previous chapter. The lipids are part of the complex deposit produced on the contact lens and are tightly adsorbed onto the surface. It is however possible to extract and analyse part of this lipoidal component of the deposit which is observed in the electrophoretic and thin layer chromatographic investigations.

The main problem with the analysis of these components in tears is the minute quantities of tear material involved. There are many of techniques for analysing biological fluids available in relatively large quantities, such as serum, urine, saliva etc.. A single contact lens surface, however, has only extremely small quantities of material deposited on it after short periods of time. Since a contact lens is often only worn for eight hours or so before removal and cleaning. This implies that little effective deposition will take place. However, after several such periods of wear and cleaning with an apparently cleaned lens,

spoilation will still eventually occur. This raises the question of whether the cleaning regimes are effective, (this point is dealt with in a later chapter), or whether an insoluble deposition process, capable of occurring within a single day is responsible for spoilation. In any case a single days wear involves the initial stages of interfacial conversion that ultimately leads to irreversible deposition and it is therefore necessary to attempt to develop a sufficiently sensitive technique to analyse these changes. This study is particularly concerned with the lipoidal species.

This chapter is therefore concerned with developing a fluorescence spectroscopy and high performance liquid chromatographic techniques to analyse the biological materials which contribute to ocular spoilation phenomena.

4.2. Development of a fluorescence spectroscopy method for contact lens analysis.

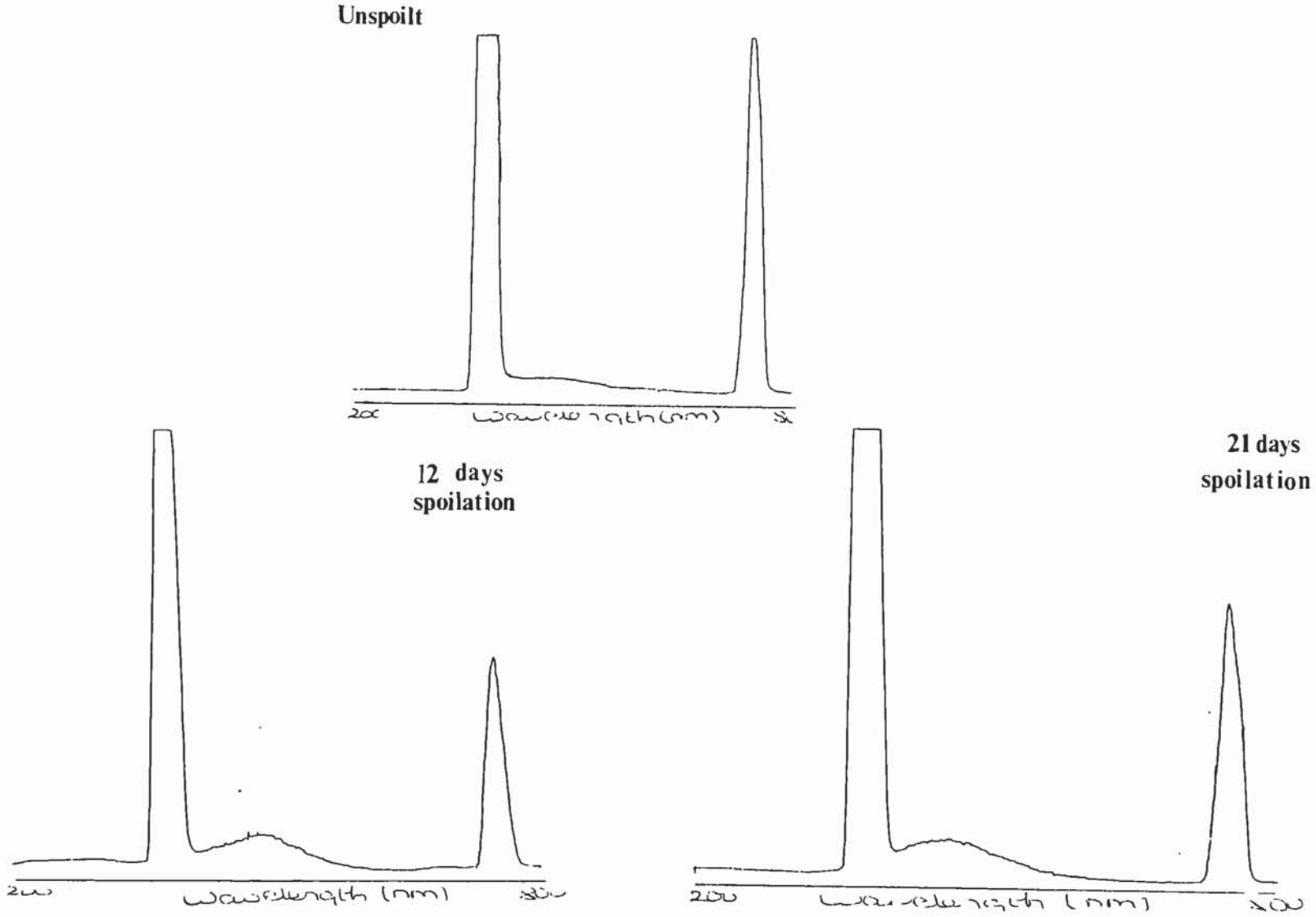
The autofluorescent nature of the lipid species was demonstrated in chapter three using fluorescence microscopy. These autofluorescent patterns are the result of intrinsic fluorophores which are present within lipids and also proteins. These fluorophores are substances which possess delocalised electrons present in conjugated double bonds. The adsorption of a photon by these electrons is followed by the emission of light from an electronically excited state. In the singlet excited state the electron in a higher energy orbital has the opposite spin orientation to the second electron in the lower orbital. These electrons are paired. The electron then relaxes to the lowest vibrational state of the excited singlet state. The excess energy being lost by vibrational relaxation in an internal conversion process. Fluorescence then occurs as the electron returns to the ground electronic state. The emitted

fluorescence energy is of a lower frequency than the absorbed energy, due to the internal conversion process [200-203].

As a result of the intrinsic fluorescent nature of lipids and proteins and because the refractive index of hydrogels and water are approximately equal, it is possible to monitor the lipids and proteins on the surface of a contact lens. In simple terms it is possible to analyse the surface of a contact lens with and without a biological layer.

Two fluorescence spectrophotofluorimeters, both Aminco-Bowman instruments were used during this study. The contact lenses are placed on a cork block in a quartz cell. The orientation of this cell within the instrument is important, in that only a single surface should be in line with light beam. Two types of spectra were run, excitation and emission. An excitation spectrum is the dependence of the emission intensity at a single wavelength, upon the excitation wavelength. An emission spectrum is the wavelength distribution of the emission, measured at a constant excitation wavelength. As with the fluorescence microscopy and to concentrate on lipid analysis, a similar excitation wavelength (i.e. 360nm) was normally used. This wavelength maximises lipid sensitivity. A typical fluorescence emission spectrum of a spoilt contact lens is shown in figure 4.1. There are three peaks; the excitation peak, emission peak and the secondary excitation peak. The excitation peak occurs at the wavelength used to excite the sample and the secondary excitation peak at approximately double the excitation wavelength. The emission peak which was termed the spoilation peak occurred around 400-600nm and was the result of biological deposition on to the contact lens surface. This peak was minimal for unspoilt lenses and could also be used to determine the presence of lipoidal or proteinaecous material in solution. The spoilation peak intensity increases with the presence of a biological layer over a period of time (figure 4.1).

Figure 4.1:- Fluorescence spectra of unspoilt and spoilt contact lenses.

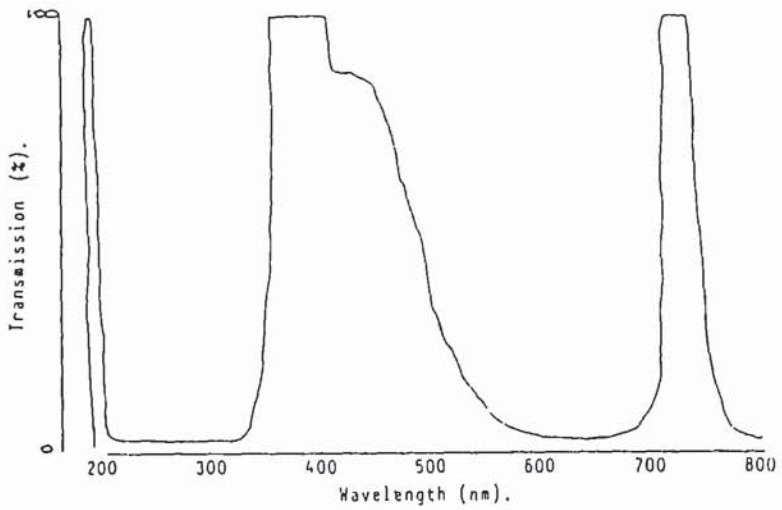


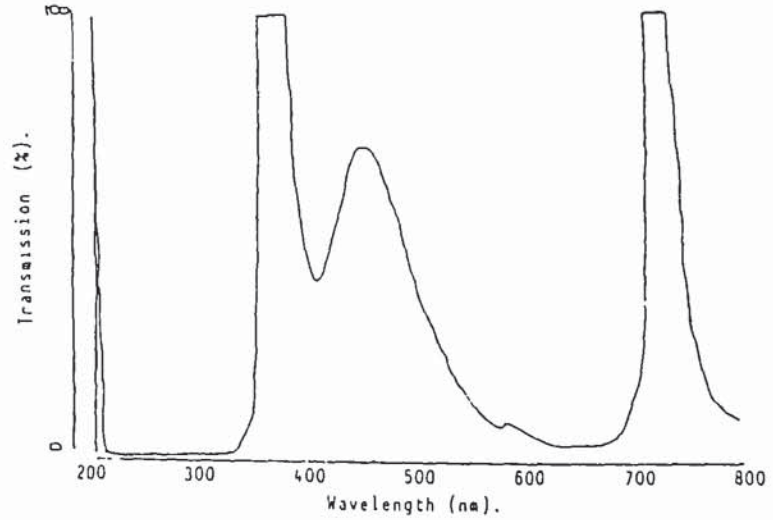
The main advantage of this technique is that it is non-destructive allowing the build-up of the biological layer on the surface of a contact lens to be monitored. As a result it is possible to use the fluorescence spectrophotofluorimeter to determine whether any biological material is deposited onto the surface of the contact lens; the extractibility of the the deposited biological layer; the extent to which the biological components are extracted and whether adsorption of any biological species occurs during the handling and cleaning of the contact lenses.

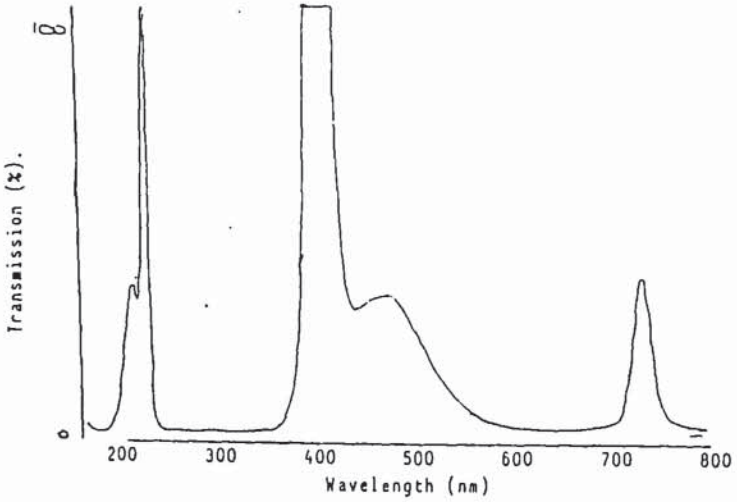
Initial studies involved the use of methanol only to extract the deposited lipids from the lenses. The next stage of the extraction developed was with sodium dodecyl sulphate (SDS) (see previous chapter). Although a decrease in fluorescence intensity occurs after the methanol and sodium dodecyl sulphate extraction (figure 4.2) and an increase in the fluorescence intensity of the solutions used for extraction occurs (figure 4.3). The post extracted lenses also showed a degree of fluorescence higher than that of an unspoilt contact lens without a biological layer (figure 4.1). This indicates that biological material is still adsorbed to the contact lens surface which are very difficult to remove or which have penetrated the matrix.

The results of these fluorescence studies show that the spectrophotofluorimeter can be used without destroying the biological layer or damaging the contact lens to study ocular spoilation phenomena. This technique also demonstrates the presence of an insoluble biological layer on the surface of the contact lens even after extraction and cleaning.

Figure 4.2:- Fluorescence spectra of methanol and sodium dodecyl extracted lenses







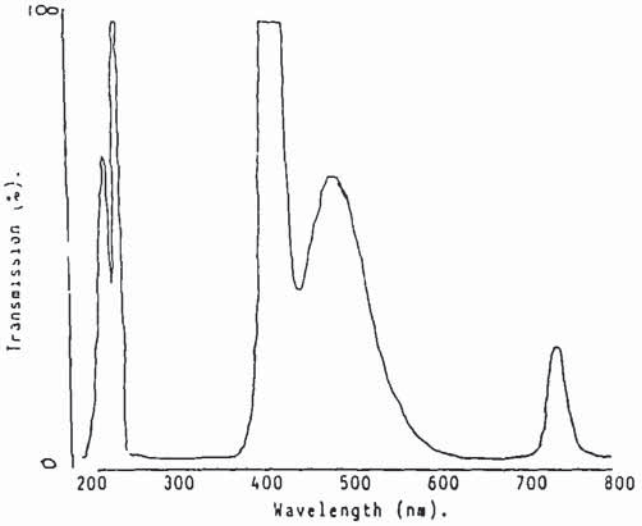
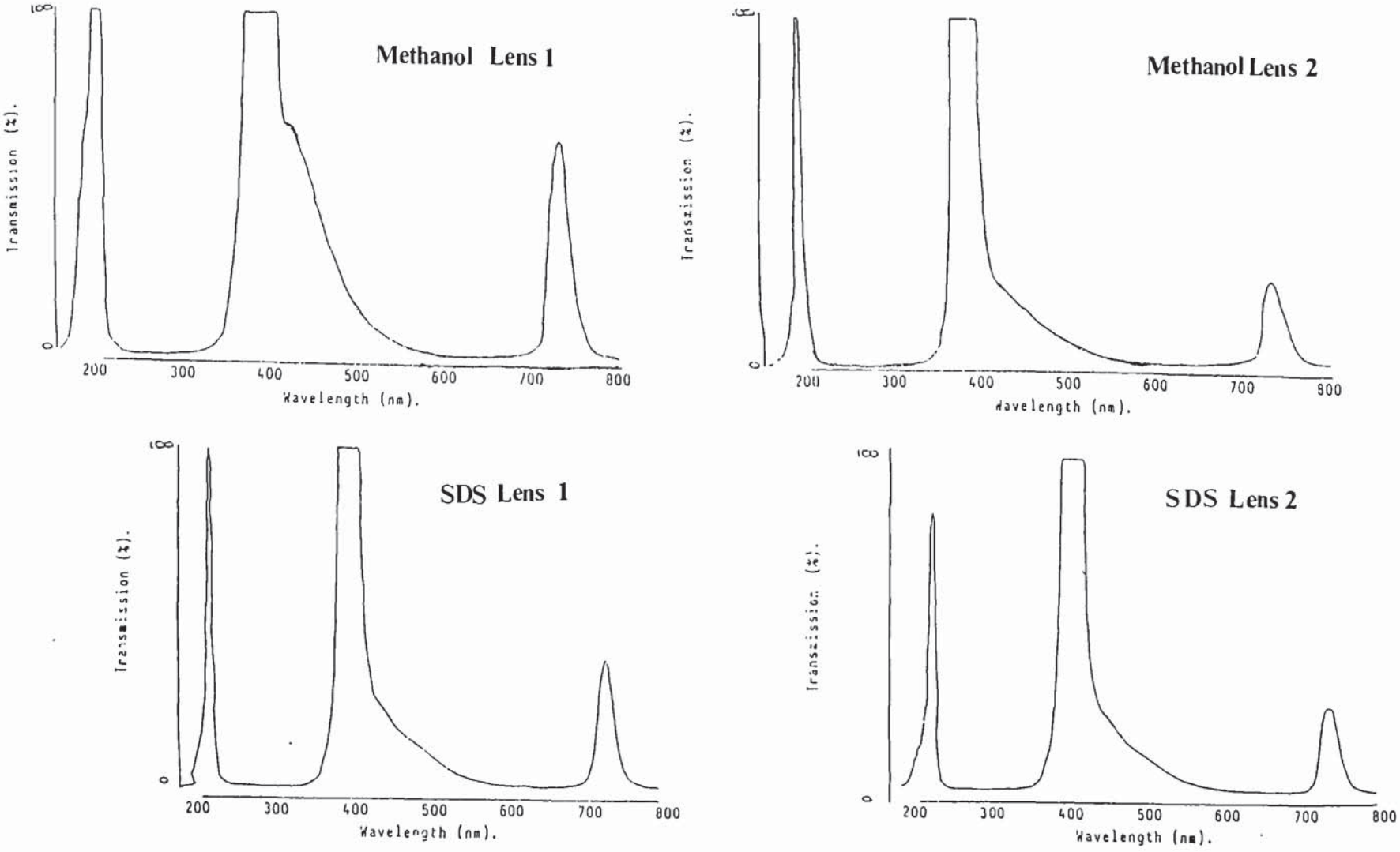


Figure 4.3:- Fluorescence spectra of methanol and sodium dodecyl extracts.



As it was possible to extract the lipids separately and high performance liquid chromatography is frequently used to separate biological solutions it was decided to develop a technique for profiling the lipids of contact lens wearers.

4.3. Development of a high performance liquid chromatographic technique.

Early work on lipid profiling of body fluids and tissues was carried out using column chromatography. Short packed gas chromatography columns have also been used for rapid lipid profiling [214]. More recently thin layer chromatography on sintered quartz rods with flame ionisation detection have been used [215]. Kuksis [217] suggested that modern HPLC systems could provide an ideal method of producing lipid profiles of body fluids and tissues. There has not been a lot of research in this area, however, because of the lack of a suitable detection method. Lipids absorb only in the short wavelength range of the UV where there is a problem choosing a suitable solvent. Classical lipid solvents such as chloroform or diethyl ether absorb strongly in the region 190nm-220nm. Trace impurities in the solvents can cause large and interfering peaks [218].

This section deals with the development of an HPLC method to build up a lipid profile of contact lens wearers. It is in part concerned with hard contact lens extraction techniques. The origins of lipoidal spoilation of worn lenses can be determined only via analysis of lenses in the early stages of deposition as has already been indicated. Therefore, analysis of a pooled sample of lenses is not sufficient. The aim was to develop a simple analytical procedure which would allow the compilation of a lipid profile from a single contact lens and hence, if possible, to identify the differences between depositors and non depositors. Surveying the literature concerned with lipids one is made aware of the diversity

of solvents, columns, mobile phases and detection methods. Some of these are summarised in table 4.1. Free fatty acids are commonly separated using a reversed phase column but detection methods often require the formation of derivatives [219-223].

Table 4.1:- Lipid separations.

Column	Mobile Phase	Detector	Reference
Spherisorb	Ternary Gradient Hexane/THF Isopropanol/Chloroform Isopropanol/Water	Mass Detector or light scattering detector	224
	(i) Hexane/Isopropanol /Acetic acid (ii) Hexane/Chlorobutane Acetonitrile/Acetic acid	UV	225
LiChrosorb SI60	Ternary Gradient CCl_4 / Isooctane $CHCl_3$/Diocan/Hexane $CHCl_3$/Methanol /Diisopropylether	moving wire	226

Table 4.1:- (continued) Lipid separations.

Column	Mobile Phase	Detector	Reference
Bondapak C	Hexane/Dichloromethane	UV	146
Porasil	Gradient	UV	227
Partisil 5	91% Hexane 9% Hexane/Isopropanol 100% Hexane/Isopropanol	UV	
8nNH_4 OH treated Spherisorb	Gradient 50% CH_3Cl 50% $CHCl_3$ 94% CH_3OH 6% NH_4OH	CIMS	228, 229

In an initial series of experiments both reversed phase and normal phase HPLC systems were tested and the eluate monitored at 210nm. Various mobile phases and extraction solvents were also investigated. The samples used were contact lenses returned from patients for reasons such as swelling, distortion and tearing. Lenses returned because of deposit formation were kept apart.

During the initial studies separations were carried out using a reversed phase column which can use methanol, acetonitrile, and water as mobile phases because of their low absorptivity in the UV. However as triglycerides are only sparingly soluble in water it is not an ideal component of a mobile phase to be used in the separation of lipid mixture

containing triglycerides. It is possible to replace the water but there may still be problems with some long chain saturated triglycerides. This chapter describes the use of a normal phase HPLC techniques to qualitatively analyse lipoidal deposition on contact lenses.

In order to analyse the materials from the lenses a suitable extraction procedure was required. A single worn lens was placed in an aluminium covered sample bottle together with 3mls of extraction solvent. The sample bottle placed on a shaker overnight. Experience showed that the extraction period could be reduced to thirty minutes. After removing the lens the extraction was concentrated to dryness with a stream of nitrogen. The residue was taken up in 300 µl of mobile phase.

The extracts were analysed using a Knauer high pressure liquid chromatograph equipped with Reodyne 7125 injector and initially with a Spherisorb S5 ODS 5µm reversed phase column (250mmx4mm ID), and the eluant detected using a UV detector. Several were examined until one with adequate baseline stability, sensitivity and resolution at the low wavelengths necessary to discriminate between the lipids and mobile phase was found. The low wavelength is the single most important factor as only a narrow window exists to allow this discrimination. This UV detector was relatively expensive to purchase and is a Perkin-Elmer LC-75 UV detector. The pumps were driven by and data collected by an Apple 11e microcomputer. This computer base also allowed some manipulation of the data collected. The computer interpretation is not always correct due to the imperfect nature of the baseline produced. Preliminary work was carried out using a Spherisorb S5 ODS 5µm reversed phase column with acetonitrile and water as a mobile phase since it was well documented that fatty acids were present in tears we expected to be able to extract them from lenses. Subsquently LiChrosorb 5 µm (250mmx4mm ID) SI 60 normal phase column was used to

allow a comprehensive lipid profile to be obtained. The UV spectrometer analysed the column eluant at 210nm. Latterly a filtered, degassed mobile phase as described by Hamilton and Comai [225] was employed. The mobile phase was hexane: propan-2-ol: glacial acetic acid 1000:5:1ml. This experimental technique was found to give excellent total lipid profiles from single contact lenses.

The initial decisions concerning hardware were made in the light of the literature available (see table 4.1). During the investigation it was decided that a second detector would add valuable insight into the problem. Fluorescence microscopic examination of worn lenses has revealed unsaturated lipoidal material including cholesterol and its esters within the deposits, hence the choice of a fluorescence detector was made. The detector chosen was a Perkin Elmer Filter LS-1 fluorescence detector (with an excitation wavelength of 360nm). Its connection in series to the UV detector enabled the comprehensive UV spectrum to be more readily interpreted, since cholesterol and cholesterol derivatives were readily identified.

Various lens handling protocols were tested prior to solvent extraction such as i) blotting dry, ii) oven drying, iii) washing in distilled water then blotting dry - this final method was found to be the optimum. A diverse array of extraction solvents can be found in the literature, but not in conjunction with the subsequent HPLC analysis [147, 161, 167, 193]. Chloroform, acetone, acetonitrile, and chloroform/hexane (1:4) appeared to extract little detectable material. Methanol, carbon tetrachloride and chloroform/methanol extractions were more effective and gave rise to similar chromatograms. Figure 4.4 shows a chromatogram acquired after extracting a single HEMA lens with 10mls of hexane overnight. Figure 4.5 is a methanol extract of a similar lens.

Figure 4.4:- Chromatogram of a HEMA lens extracted with 10mls hexane overnight.

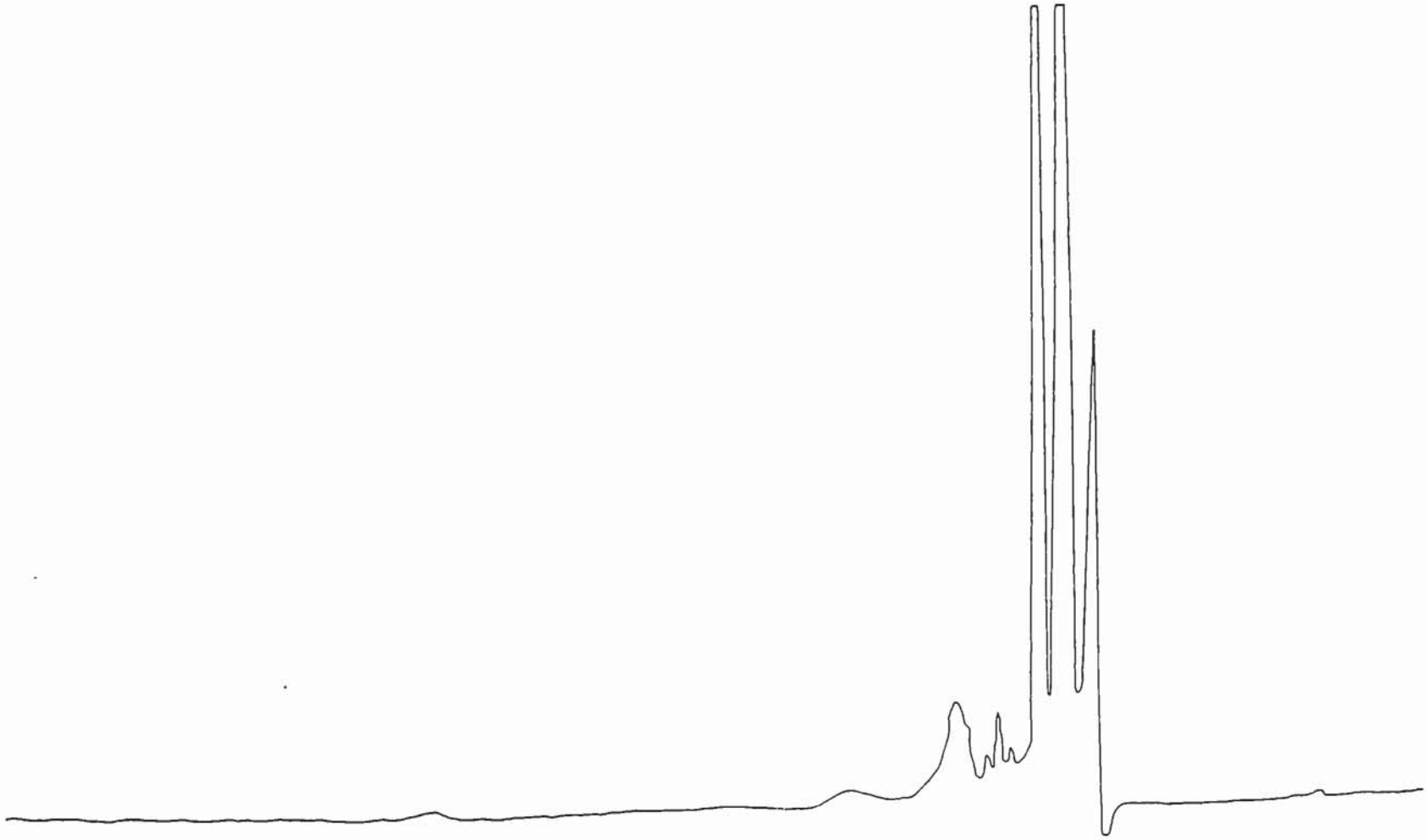
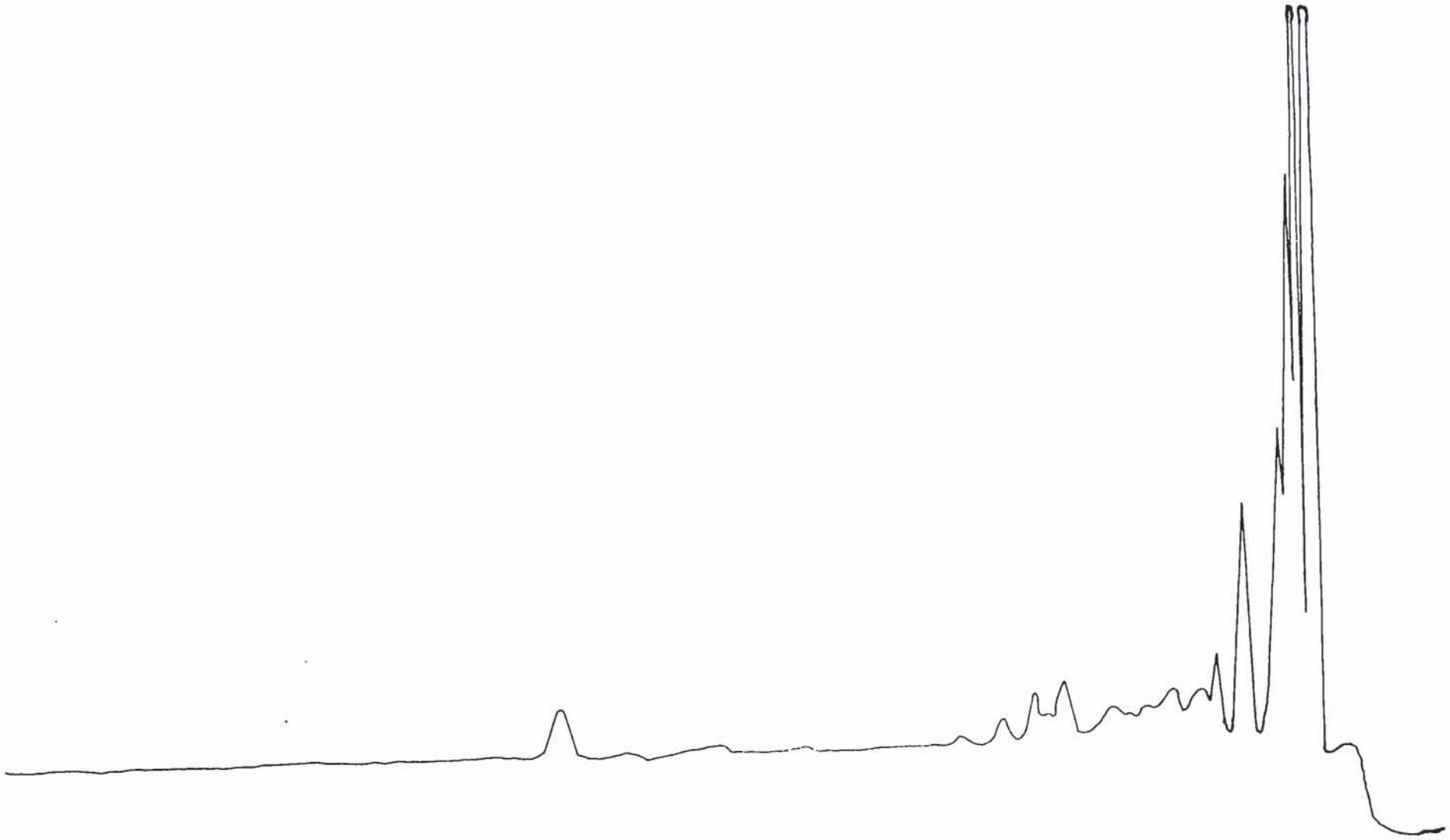


Figure 4.5:- Chromatogram of a HEMA lens extracted with 10mls methanol overnight.



Peaks were identified by comparing their retention times with those of knows standards (table 4.2).

Table 4.2:- Retention times of the HPLC lipid standards.

Lipid	Rt	Rt range	Rt range of lipid class
Cholesterol esters:-			
Cholesterol linoleate	69	68-73	
Cholesterol oleate	68	66-72	61-73
Cholesterol palmitate	67	60-66	
Glycerides:-			
Monostearin	603	503-727	503-727
Dipalmitin	236	202-260	202-260
Triolein	114	110.67-118	110-118
Trilaurin	146	136-163	
Fatty acids:-			
Arachidic acid	177	174-189	
Linoleic acid	279	274-281	
Linolenic acid	278	265-288	170-288
Oleic acid	190	181-208	
Palmitic acid	179	170-186	
Stearic acid	174	171-179	

Table 4.2:- (continued) Retention times of the HPLC lipid standards.

Lipid	Rt	Rt range	Rt range of lipid class
Phospholipids:-			
L- α-phosphatidylcholine	198	187-235	
L- α-phosphatidylinositol	72	71-75	
	2381	229-248	71-118
L-α -phosphatidyl-L-Serine	115	112-118	127-248
	132	127-141	
Cholesterol:-			
Cholesterol	907	862-941	862-942
Lipid standard:-			
Cholesterol oleate	667	6668	66-68
Triolein	109	102-114	102-114
Oleic acid	292	288-295	288-295
Cholesterol	929	868-973	868-973

A variety of extraction solvents listed previously which include hexane, carbon tetrachloride, methanol, acetonitrile, acetone, and chloroform have all been mentioned in the literature as lipid extraction solvents. In this study we tested pure, chlorinated and nonchlorinated solvents and mixtures. Chloroform and dichloromethane were found to absorb so strongly in the detection region that they masked any extracted material. Chao and Vergnes [230] reported that extraction with a 2:1 v/v chloroform/methanol removed nearly 90% of the total lipid which then was separated by thin layer chromatography. Of the other solvents tested it was found methanol was the most efficient in removing lipoidal material from the contact lenses. Methanol is also the most successful lipid solvent for extracting lipids from the interfacial layer deposited onto polystyrene during cell culture experiments[231].

A hydrophilic solvent may be able to make better contact with the lens and therefore be able to solvate more deposited material. A hydrophobic solvent will make a large contact angle with the lens and so not make intimate contact with a hydrated lens. It is also essential for the extraction solvent to be compatible with the mobile phase used.

The efficiency of the extraction method was tested by carrying out a second solvent extraction and chromatographing the extract. A decreased quantity of UV absorbing material was detected with a second extract of either methanol or hexane. The amount of lipoidal material extracted by the second extract was 18% of the original quantity removed by the primary methanol extraction. With the second extraction using hexane, after an initial methanol extraction, 30% of the original quantity of the lipoidal material removed by the primary methanol extraction. Despite the use of a second organic solvent, some residual fluorescence remained on the lens surface. This residual fluorescence was decreased by 1%

transmission with hexane used as the second extraction solvent, whereas with the second extraction using methanol showed no further decrease in the fluorescence intensity on the lens. The fluorescence remaining on the lenses after the second extraction with an organic solvent could be further reduced by extraction with sodium dodecyl sulphate (SDS). This fluorescence was caused by protein present on the lenses. A residual fluorescence intensity remained on the lens surface even after this extraction with SDS suggesting that some lipoidal material is polymerised to the lens surface or that lipid (e.g. fatty acids) may have penetrated the lens matrix.

The specificity of the extraction method was also tested; after a 30 minute solvent extraction the lenses were placed in a 1% SDS solution and shaken for 30 minutes. Both the methanol and the SDS extracts were run on SPE Paragon (protein) electrophoresis gels. The methanol extract showed no positive protein presence while protein, was as expected found in the SDS extract.

4.4. Discussion of results.

The results of these preliminary studies have produced suitable sensitive technique to allow the analysis of the lipoidal deposition and produced vital new information in tear composition studies. The fluorescence spectrophotofluorimeter can be used to monitor the progression of spoilation on the contact lens without destroying the biological layer or damaging the contact lens. It is also useful in determining how much biological material can be removed from the contact lens using extraction and cleaning techniques. The high performance liquid chromatography technique which can analyse single contact lens is a major advance on previous work [146,148] which has centred on the use of a pooled supply of

lenses returned because of deposit formation. This leads to differences in the detected tear components due to individual variations in tear chemistry and poor analytical techniques.

This variation in perceived composition is demonstrated in the literature. Hart [148] claimed that cholesterol while present in the tear film was present in deposits only as long chain esters. He considered deposit formation to be similar to that of the Meibomian gland secretions except that there were higher levels of triglycerides present in the deposits. Rapp[146] was unable to detect any cholesterol, cholesterol esters or triglycerides in lens extracts.

The development of these two techniques was vital to allow the analysis of the minute quantities involved in ocular spoilation. These techniques were then used to analyse *in vivo* and lenses which were spoilt using an *in vitro* model developed during this study (see chapter six).

CHAPTER 5.

Analysis of individual spoiled contact lenses by high performance liquid chromatography (HPLC) and fluorescence spectroscopy.

5.1. Introduction.

The two techniques described in the previous chapter are now used routinely in these laboratories in the analysis of spoiled contact lenses. The purpose of this chapter is to show the applicability and sensitivity of the two techniques, particularly high performance liquid chromatography (HPLC) in the analysis of contact lenses from clinical sources. The previous chapter has shown the way in which high performance liquid chromatography has been developed into a technique that is suitably sensitive to allow the analysis of lipoidal deposition on a single contact lens. In order to examine whether these techniques are capable of the sort of detailed analysis required for use in parallel with clinical studies a series of investigations were carried out.

This chapter describes these investigations using a small number of representative chromatograms, from over two hundred recorded during this study, to describe the results obtained. In particular the chapter deals with the spectrum of lipoidal deposition that occurs on hard lenses and on soft lenses in particular noting any differences that may occur between left eye and right eye of the same patient; it deals with lipoidal depositions associated with discrete deposits both elevated white spots and calcium films; it examines the possibility of use of the techniques for very short term (approximately 15 minutes) insertion as a means of sampling the 'tear envelope' and finally deals with contamination from extrinsic sources such as skin lipids. In these studies the fluorescence spectroscopy and dual detector (UV and fluorescence) high performance liquid chromatography (HPLC) were frequently used together in analysis.

The specific advantage of fluorescence spectroscopy is that it enables the non-

destructive analysis of the whole lens or of a solution which has been used to extract the deposit from the lens. It gives a quantitative assessment of the fluorescent components present on the lens or in the solution and allows that lens to be used further either clinically or in further extraction/analytical studies. It is not, however, capable of separating individual fluorescent components or providing any detail as to their origin. High performance liquid chromatography on the other hand is destructive in the sense that the film of deposited material is extracted from the lens surface. It does, however, allow reasonably complete analysis of the lipoidal components present on the surface. If the solvent extraction used does not cause distortion or damage to the lens, that lens can then be used further for clinical studies. The great value of these two techniques taken together is that they indicate quantitatively the amount of lipoidal/fluorescent material on the lens surface, by extraction they allow its detailed analysis and by fluorescence spectroscopy allow determination of any fluorescent material which remains on the lens surface.

Following the developmental high performance liquid chromatography studies a standard protocol was adopted and all the contact lenses were analysed in the same way. A single contact lens was extracted in 3ml of methanol on a shaker for 30 minutes, after which time the lens is removed and the extract dried down under nitrogen. The sample was then frozen prior to use. The samples were then re-dissolved in 200µl of mobile phase (hexane : propan-2-ol : acetic acid 1000 : 5 : 1 ml); 50µl of which was then injected onto the column. The chromatograms were run for 30 minutes. Each extract was run at least three times and the average retention time calculated. These chromatograms were then compared to a series of standard lipids which had been used to calibrate the HPLC column used during this study (see chapter 4). It is possible to identify the class of lipid, but not

individual components due to peak overlap and the shift in retention time which occurs due to column loading when analysing mixed solutions.

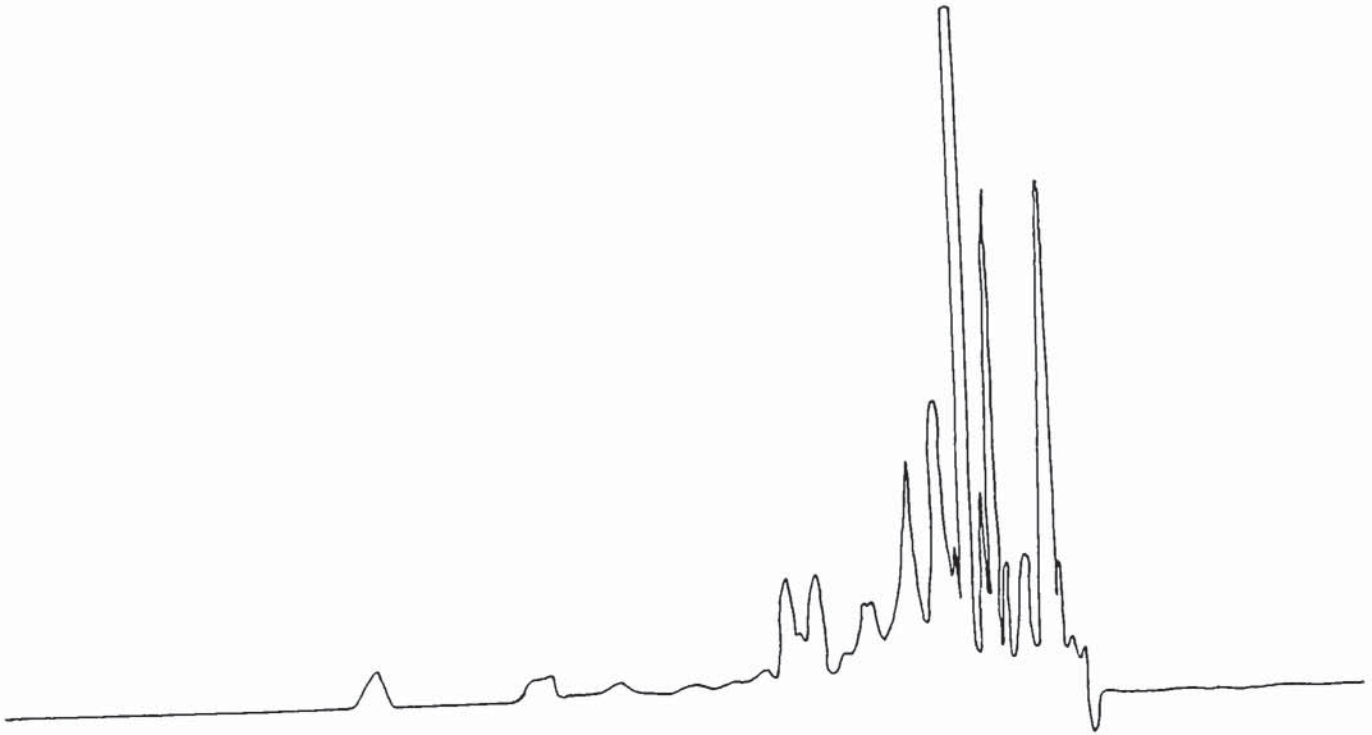
Although the column was calibrated, the concentrations of the individual lipoidal components are difficult to calculate. This is because quantification of lipids using low wavelength UV detection is not possible unless the number of double bonds is constant and known [218]. This fact results in the same concentration of for example fatty acids yielding calibration curves of different areas for the same concentration [225]. Also some compromise was necessary in the choice of column/mobile phase. The advantages of a single column and separation procedure that allows the various lipoidal families to be finger printed are obvious. They do however entail some lack of separation within each of the classes. Using different column conditions complete separation of the fatty acid family can for example be achieved be with virtually no resolution of triglycerides and cholesterol esters[232, 233]. Early work in this project developed an extremely sensitive separation for tear-derived fatty acids, illustrated in figure 5.1. Since the essence of the analysis of a single contact lens was the use of a simple single analytical technique to determine the relative effects of patient and material variables it was clearly inappropriate to chose a series of protocols to achieve excellent separation of each component. Consequently, in choosing a technique that will provide a total lipid 'finger print' there is inevitable compromise in obtaining individual retention times of each member of each of the lipid classes and a problem in resolving the areas of different components of the same lipid class. It is however possible to estimate the quantity of material within each class. In cases where it is desirable to obtain a complete analysis of individual members of a family alteration of the analytical conditions and careful analysis of the chromatograms does permit very reasonable estimation of individual members of a class. It is inconvenient to have to do this

for each run.

This chapter presents in sequence with the type of result obtained from contact lens insertion for a short time period to sample the 'tear envelope'; analysis of soft contact lenses with and without white spot deposits; calcium film deposited lenses; hard contact lenses worn on a daily basis for longer time periods and the effect of extrinsic contamination by skin lipids. The intention is to present the baseline which could be used for detailed analysis in parallel with carefully planned clinical control of patients to isolate specific effects and variations.

The chromatograms shown are representative of the high performance liquid chromatography traces recorded. The Y-axis is the peak height (mV) and the X-axis the retention time in minutes. The chromatograms were run for thirty minutes and the retention times of the peaks are given in the tables prior to the chromatograms. This is considered to be the most satisfactory form of representation in view of the necessary break in the time axis of each chromatogram.

Figure 5.1:- Chromatogram of free fatty acids.



5.2.1. Contact lens insertion for short time periods to sample 'tear envelopes'.

Studies during the development of the high performance liquid chromatography technique described in chapter 4 indicate that the analysis of the minute quantities of lipoidal species on contact lenses is possible. It is however also necessary to analyse the initial interaction between the contact lens and tear film. As a result this high performance liquid chromatography technique was used to analyse the tear components present in the human tear film. This was necessary due to the lack of definite information on the detailed composition of human tear film lipids, which is due to their low concentrations. This can be achieved by inserting a contact lens in the eye for a short period of time and analysing the resulting tear fluid envelope removed with the contact lens. Soft and hard contact lenses were tested after a 15 minute insertion period using a standard protocol described below.

The contact lenses were sterile when inserted into the eye and handled only with gloves to prevent any material being deposited onto the lens. The lenses were then placed into sterile sample bottles without contacting with any liquid prior to their extraction with methanol. This means that the material removed from the contact lens should be equivalent to the tear film of the patients sampled. The contact lenses are from six different patients. The use of the standard protocol was necessary to minimise the variation which might have otherwise occurred due to factors other than variation in patient tear chemistry. Such factors include tear flow, loss of components onto the glassware used during analysis and the effect of the variation in concentration, some components concentrations being very low.

The results for four soft lenses (two right eye and two left eye lenses) and two hard lenses (right and left) are given below.

The right eye lenses show clear well separated chromatograms (figures 5.1, 5.3) with cholesterol esters, triglycerides, fatty acids and other lipid classes. There is relatively little variation in the pattern of peak areas produced, in that the triglycerides have the greatest area followed by the cholesterol esters, fatty acids and other lipid classes. The total (i.e. of all lipid classes) peak areas varies very little from lens to lens.

The left eye lenses also show well formed chromatograms (figures 5.4, 5.5) with cholesterol esters, triglycerides, fatty acids, cholesterol and other lipid classes. There is some variation in the pattern of peak areas observed with the left eye lenses. The first patient shows a decrease in concentration triglyceride, cholesterol esters, fatty acids, cholesterol and other lipid classes whereas the second shows a decrease triglyceride, cholesterol esters, cholesterol, fatty acids and other lipid classes. The total lipid concentration assessed from cumulative peak areas are, however, similar.

The two left eye chromatograms and two right eye chromatograms are fairly similar for each eye in nature. The differences between the eyes is however greater as both left eyes show a cholesterol component whereas both the right eyes do not.

Table 5.1:- Rt times of the lipid extracts of soft contact lenses worn for 15 minutes.

Lens	Rt	Identity	Lens	Rt	Identity
Right 1	66	Cholesterol ester	**Right 2**	61	Cholesterol ester
	72	Cholesterol ester		68	Cholesterol ester
	75	Cholesterol ester		77	Cholesterol ester
	167	Triglyceride		101	Triglyceride
	184	Triglyceride		128	Triglyceride
	205	Fatty acid		147	Fatty acid
	299	Fatty acid		155	Fatty acid
	470			244	Fatty acid
	520			615	
Left 1	60	Cholesterol ester	**Left 2**	60	Cholesterol ester
	68	Cholesterol ester		68	Cholesterol ester
	81	Cholesterol ester		80	Cholesterol ester
	126	Triglyceride		116	Triglyceride
	141	Triglyceride		153	Triglyceride
	172	Triglyceride		171	Triglyceride
	216	Fatty acid		250	Fatty acid
	358			325	
	800	Cholesterol		798	Cholesterol

Figure 5.2:- Chromatogram of right eye soft contact lens 1 inserted for 15 minutes.

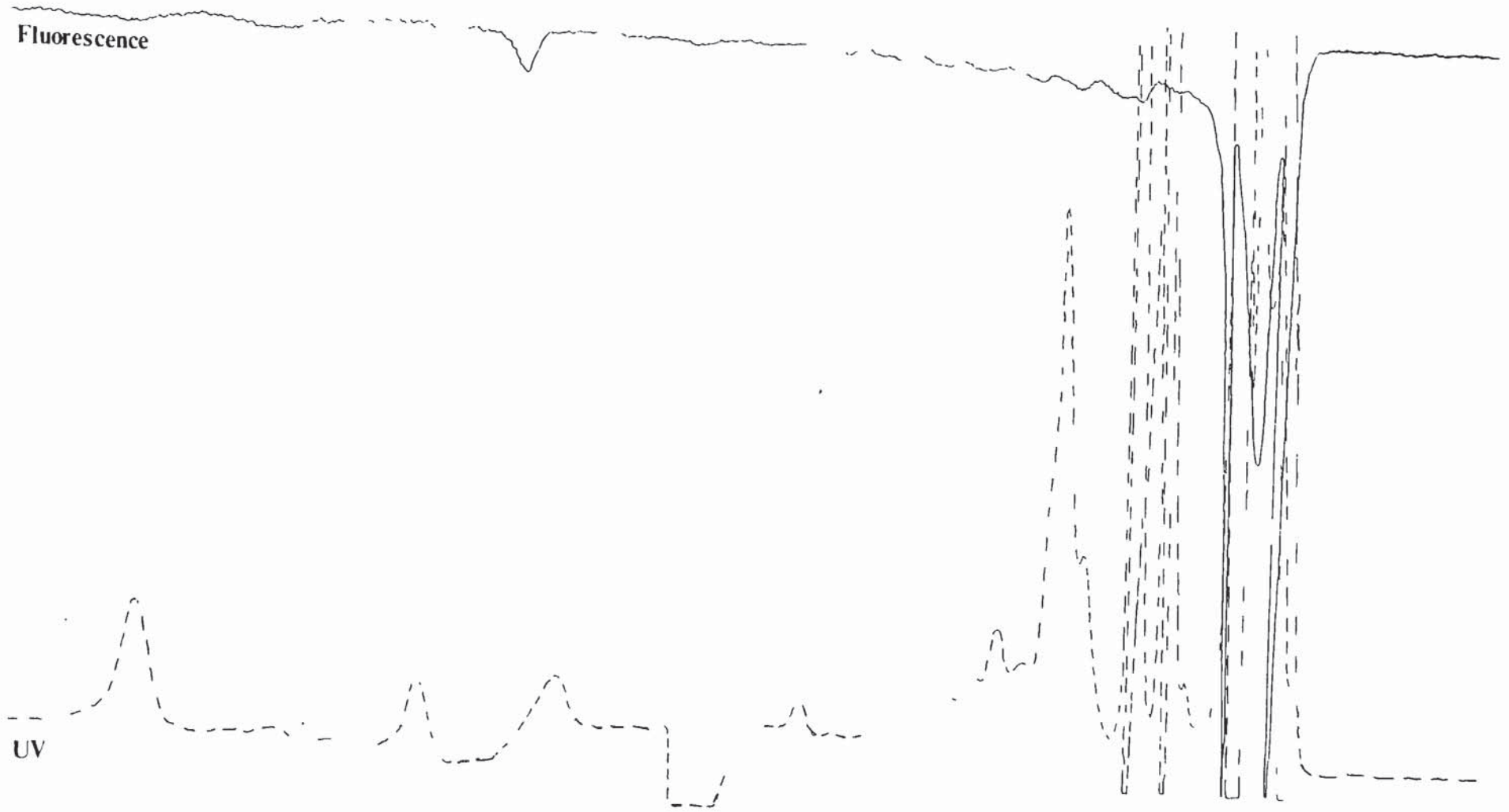


Figure 5.3:- Chromatogram of right eye soft contact lens 2 worn for 15 minutes.

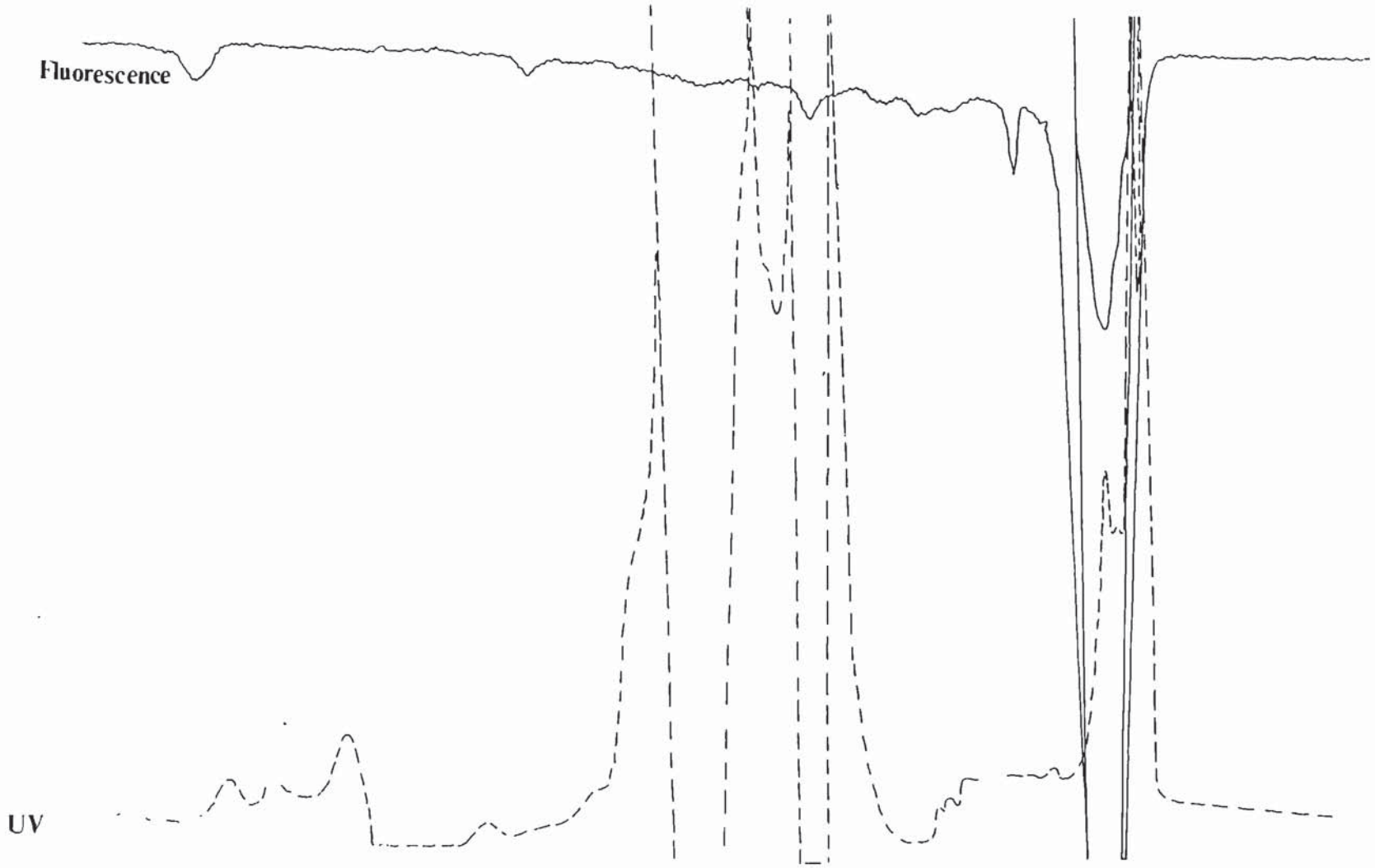


Figure 5.4:– Chromatogram of left eye soft contact lens 1 inserted for 15 minutes.

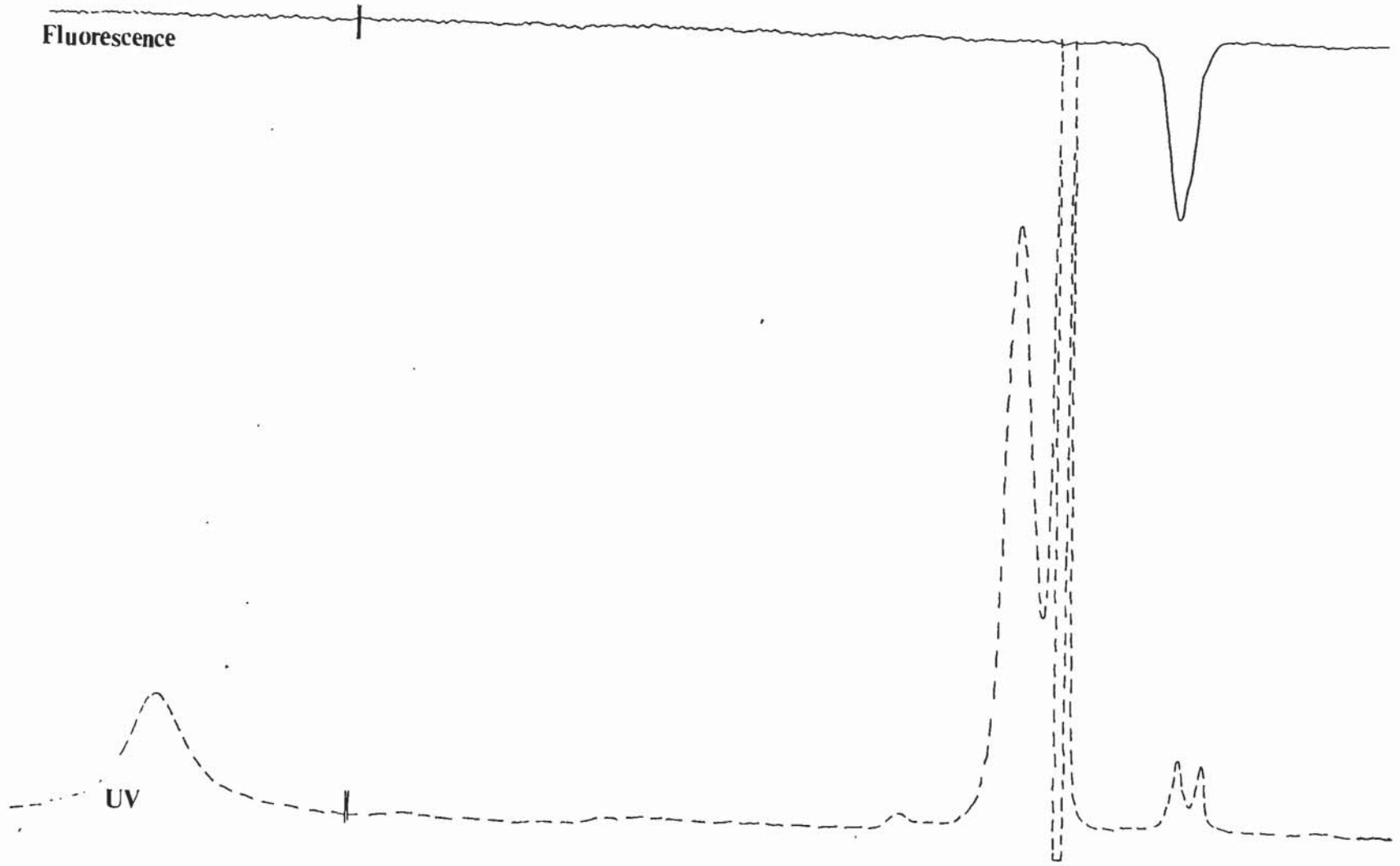
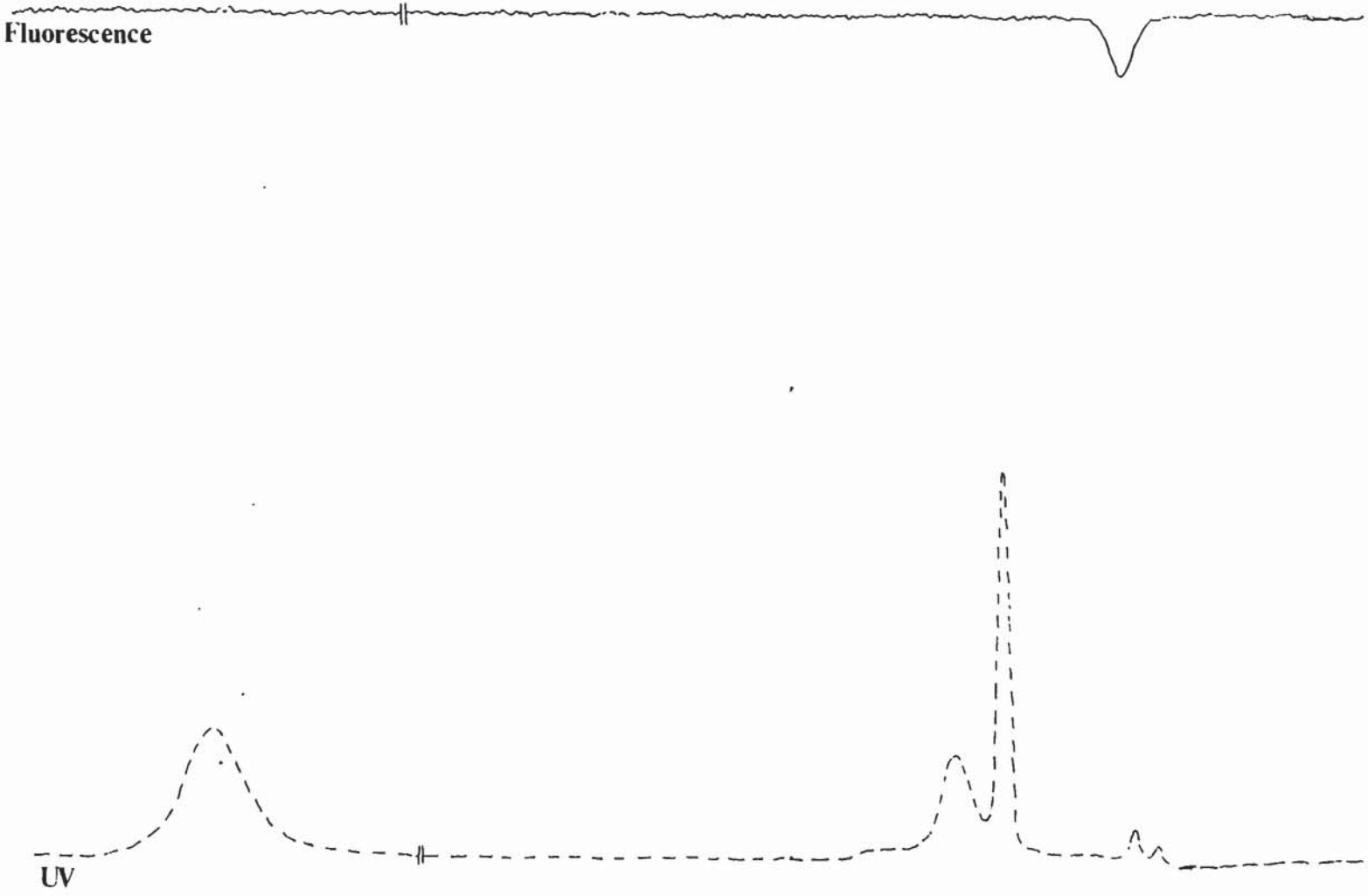


Figure 5.5: Chromatogram of left eye soft contact lens 2 worn for 15 minutes



The two hard contact lenses used for the tear envelope sampling were Boston lenses (siloxy-methacrylate-itaconate copolymer). Both these hard lenses show well formed chromatograms (figures 5.6, 5.7). The retention times are indicative of cholesterol esters, triglycerides, fatty acids, cholesterol and other lipid classes. It is also likely that some of this material is indicative of diglycerides, monoglycerides, fatty alcohols and phospholipid. The peak areas follow a pattern which resembles that of the soft lenses (triglycerides, cholesterol esters, fatty acids, cholesterol and other lipid classes), although the difference in peak area between the triglycerides and cholesterol esters is smaller. The total lipid peak areas of the hard contact lenses are however similar and are also comparable to the soft lenses. (It is interesting to note that the Boston lenses worn for 18 months were also worn by this patient. The extracted material from the lenses worn for 18 months represents only a proportion of the number of components collected after 15 minutes - see section 5.2.5).

From a superficial examination the use of a lens insertion technique to sample total tear envelopes was apparently successful and showed no sampling variation between hard and soft results.

Table 5.2:-Retention times of the lipid extracts of hard contact lenses worn for 15 minutes.

RIGHT		LEFT	
Rt	Possible identity	Rt	Possible identity
66	Cholesterol ester	67	Cholesterol ester
79	Cholesterol ester	78	Cholesterol ester
84	Cholesterol ester	84	Cholesterol ester
98	Triglyceride	99	Triglyceride
104	Triglyceride	101	Triglyceride
114	Triglyceride	110	Triglyceride
133	Triglyceride	129	Triglyceride
214	Fatty acid	200	Fatty acid
23	Fatty acid		
288	Fatty acid	294	Fatty acid
340		323	
429		433	
		638	
710	Cholesterol	733	Cholesterol

Figure 5.6:- Chromatogram of a left eye hard contact lens inserted for 15 minutes.

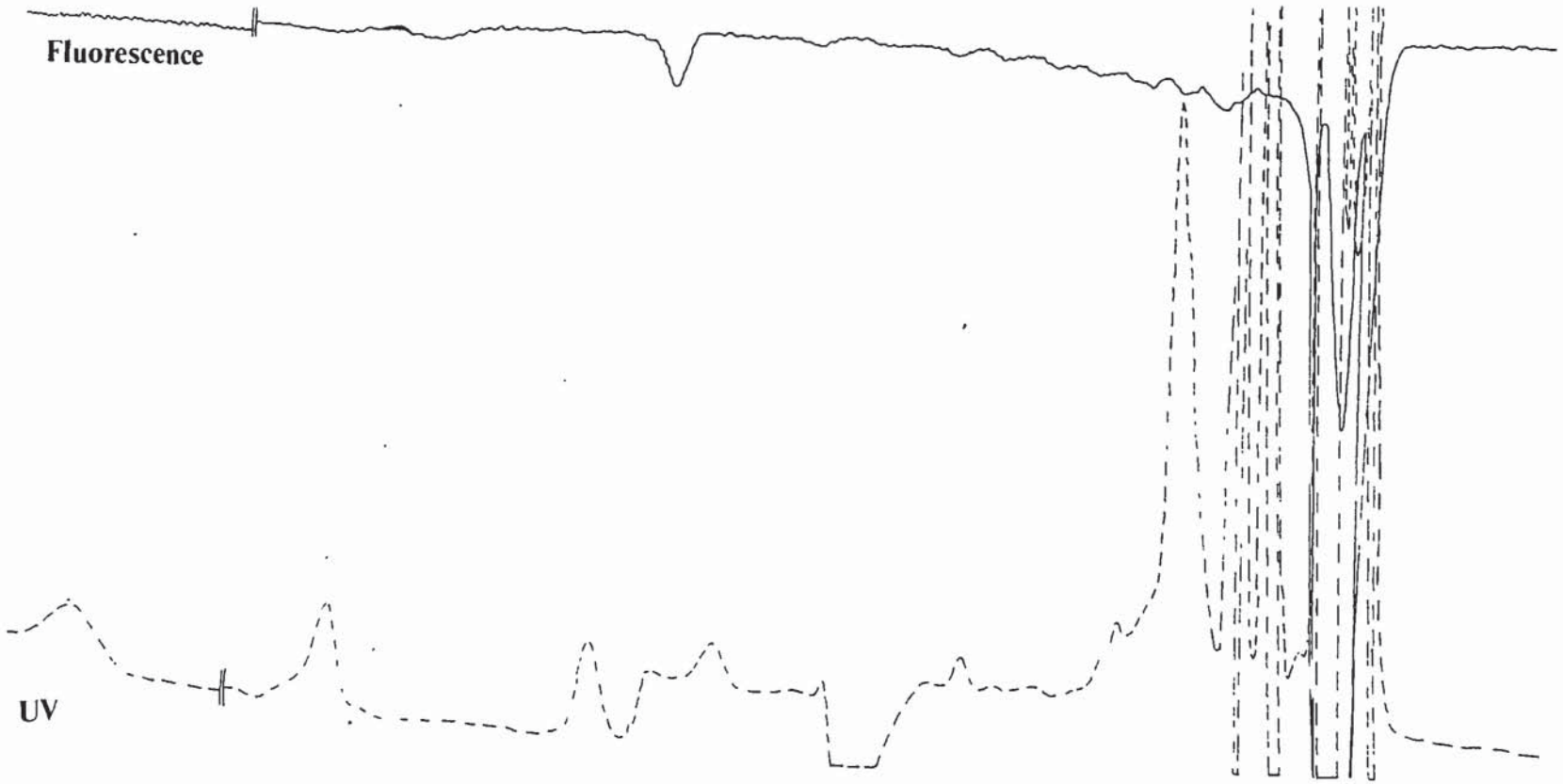
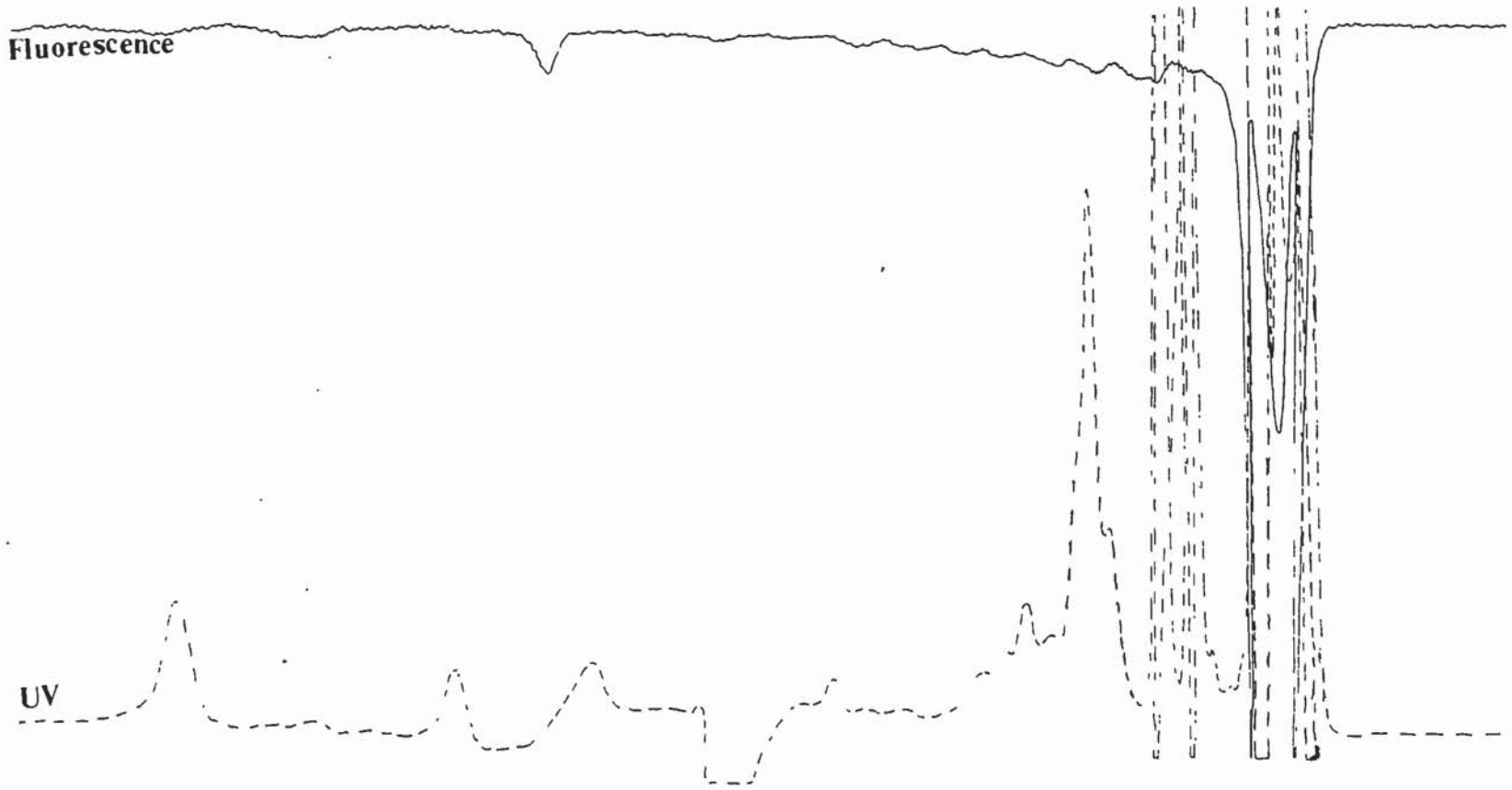


Figure 5.7:- Chromatogram of a right eye hard contact lens inserted for 15 minutes.



5.2.2. Analysis of soft lenses with white spot deposits.

An initial investigation was carried out on lenses that showed spoilation phenomena as a result of prolonged wear. The pattern of lipoidal deposition on two high water content extended wear contact lenses that had produced white spot deposits. One Permaflex lens (copolymer of methyl methacrylate, vinyl pyrrolidone and other methacrylate, 74 % equilibrium water content) and one X-ten lens (complex copolymer of five different monomers; the principal additional ones being amide-based). The two lenses were extracted separately using methanol. White spot deposits were still present on the lens surfaces after extraction. The chromatograms produced from the methanol extracts of the lenses show well defined patterns (figures 5.8, 5.9).

The Permaflex lens showed peaks which correspond to cholesterol esters, triglycerides, fatty acids and other lipid classes. The component eluted around 605 seconds is likely to be a monoglyceride or fatty alcohol. Phospholipids may also be present as the retention time for this class is masked by the retention times for the other lipid classses. The chromatogram for the X-ten lens shows a pattern of retention times which resembles that of the Permaflex lens. The peaks indicate the presence of cholesterol esters, triglycerides, fatty acids and other lipid classes.

Both soft contact lenses show retention times which correspond to cholesterol esters, triglycerides, fatty acids and other lipid classes. A phospholipid and diglyceride component may also be present as the retention times of these classes fall within the ranges covered by the cholesterol esters, triglycerides and fatty acids. The peak areas follow a pattern which is comparable to the lenses inserted for 15 minutes. The greatest area being

the triglycerides followed by the cholesterol esters and fatty acids and lastly the other lipid classes. The total lipid peak area for the two soft lenses were similar.

Table 5.3:-Rt times of the lipid extracts of the white spot deposited lenses.

Lens	Pk	Average Rt time	Rt range	Possible identity
Permaflex	1	69	68-69	Cholesterol ester
	2	71	71-72	Cholesterol ester
	3	76.	76-77	Cholesterol ester
	4	104	104-105	Triglyceride
	5	111	109-112	Triglyceride
	6	122	121-123	Triglyceride
	7	258	246-270	Fatty acid
	8	605	600-620	
X-ten	1	70	68-72	Cholesterol ester
	2	73	71-85	Cholesterol ester
	3	122	120-125	Triglyceride
	4	180	180	Fatty acid
	5	617	600-630	

Figure 5.8:- Chromatogram of a Permaflex lens.

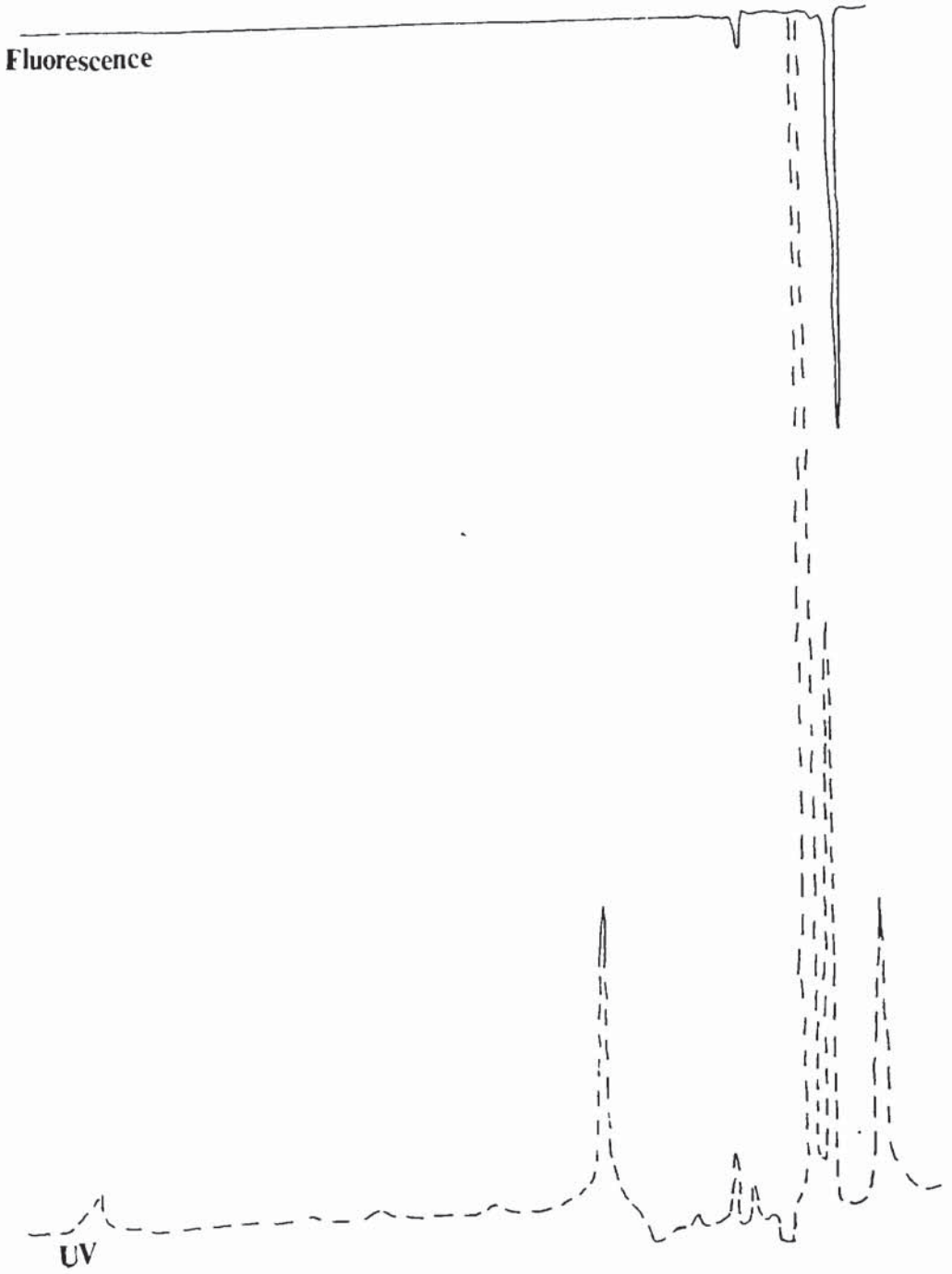
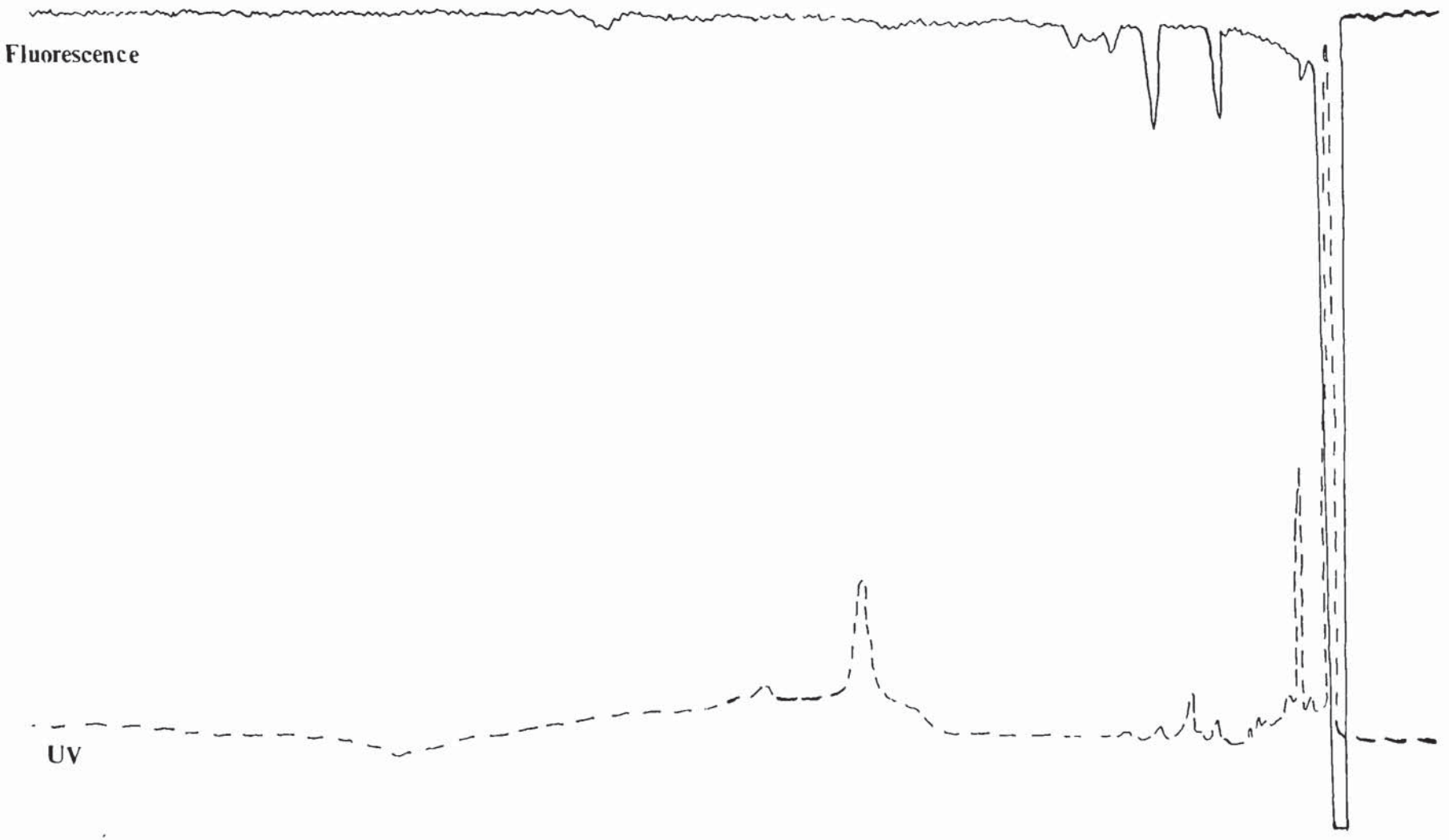


Figure 5.9:- Chromatogram of an X-ten lens.



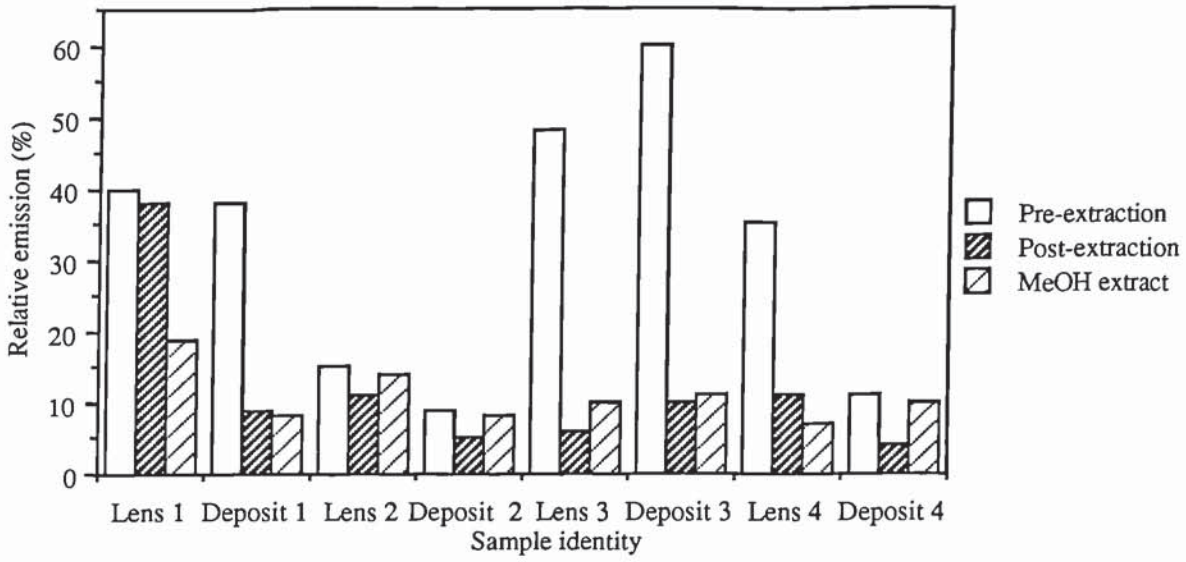
After studying the extractable lipoidal material from deposited contact lenses it was decided to attempt to determine any differences between the lipids extracted from the white spot and those extracted from the lens surface.

5.2.3. White spot deposit and lens extraction.

Four lenses with white spot deposits were used for this investigation; two Permaflex (copolymer of methyl methacrylate, vinyl pyrrolidone and other methacrylate, 74% water content) and two X-ten lenses (complex polymer of five different monomers; the principal additional ones being amide-based). The white spot deposits were excised from the contact lenses and then separately extracted from the remaining lens sample. Preliminary observations revealed that for all the lenses the greatest fluorescence intensity of excitation was obtained at 360nm. As a result the emission spectra were all run at this wavelength. Fluorescence spectra were recorded prior to and after extraction with methanol for all the white spot and lens samples.

The white spot deposited lenses and the remaining lenses after excision of the white spots when analysed separately both had fluorescent components, as shown by the peak between 400-600nm on the fluorescence spectra. For all the lenses analysed a decrease in the fluorescence intensity is observed which is indicative of the extraction of organic material from the contact lens. It was however difficult to mount the white spot deposits for fluorescence analysis. Both the unspoiled area of the contact lens and white spot areas had fluorescent material present on the surface after the extraction of the lipoidal element with methanol.

Figure 5.10:- Fluorescence intensity values of the lens and white spot deposit samples.



After extraction the fluorescence intensity value of the methanol had increased, which is another indication that lipoidal material has been removed from the contact lenses and white spots. Each white spot and lens methanol extract was run for 30 minutes and compared to the lipid standards.

Table 5.4:- Retention times of the lipoidal material extracted from the contact lenses and the white spot deposits.

Lens	LENS		WHITE SPOT	
No.	Rt	Possible identity	Rt	Possible identity
1	68	Cholesterol ester	68	Cholesterol ester
	79	Cholesterol ester	79	Cholesterol ester
	100	Triglyceride	112	Triglyceride
	142	Triglyceride		
	167	Fatty acid		
	241	Fatty acid	237	Fatty acid
	651		636	
2	67	Cholesterol ester	67	Cholesterol ester
	76	Cholesterol ester	73	Cholesterol ester
			92	Triglyceride
	107	Triglyceride	115	Triglyceride
	144	Triglyceride	133	Triglyceride
	155	Triglyceride		
	752	Cholesterol	623	

Table 5.4:-(continued) Retention times of the lipoidal material extracted from the contact lenses and the white spot deposits

Lens	LENS		WHITE SPOT	
No.	Rt	Possible identity	Rt	Possible identity
3	67	Cholesterol ester	70	Cholesterol ester
	76	Cholesterol ester	80	Cholesterol ester
	104	Triglyceride		
	128	Triglyceride	127	Triglyceride
			685	
	755	Cholesterol		
4	66	Cholesterol ester	70	Cholesterol ester
	76	Cholesterol ester	80	Cholesterol ester
	119	Triglyceride	120	Triglyceride
			130	Triglyceride
	872	Cholesterol	680	

The contact lens samples show a greater number of extracted lipids than the excised white spot deposits. There is a variation in the lipids extracted from the contact lenses and excised white spot deposits (figures 5.11-5.18). The peak areas for the contact lens samples and excised white spot deposits show a variation in the observed relative quantity pattern. The contact lens samples 1, 2 and 3 all show cholesterol esters,

triglyceride, fatty acids, cholesterol and other lipid classes pattern for both the lens and excised white spot deposit. Contact lens sample 4, however, shows the pattern triglyceride, cholesterol esters, cholesterol and other lipid classes. The total lipid peak areas for the contact lens samples 1, 2 and 3 is greater for the lens than the excised white spot deposit, whereas contact lens sample 4 has a total lipid peak area which is greater for the excised white spot deposit than the lens sample.

Figure 5.11:- Chromatogram of soft lens sample 1.

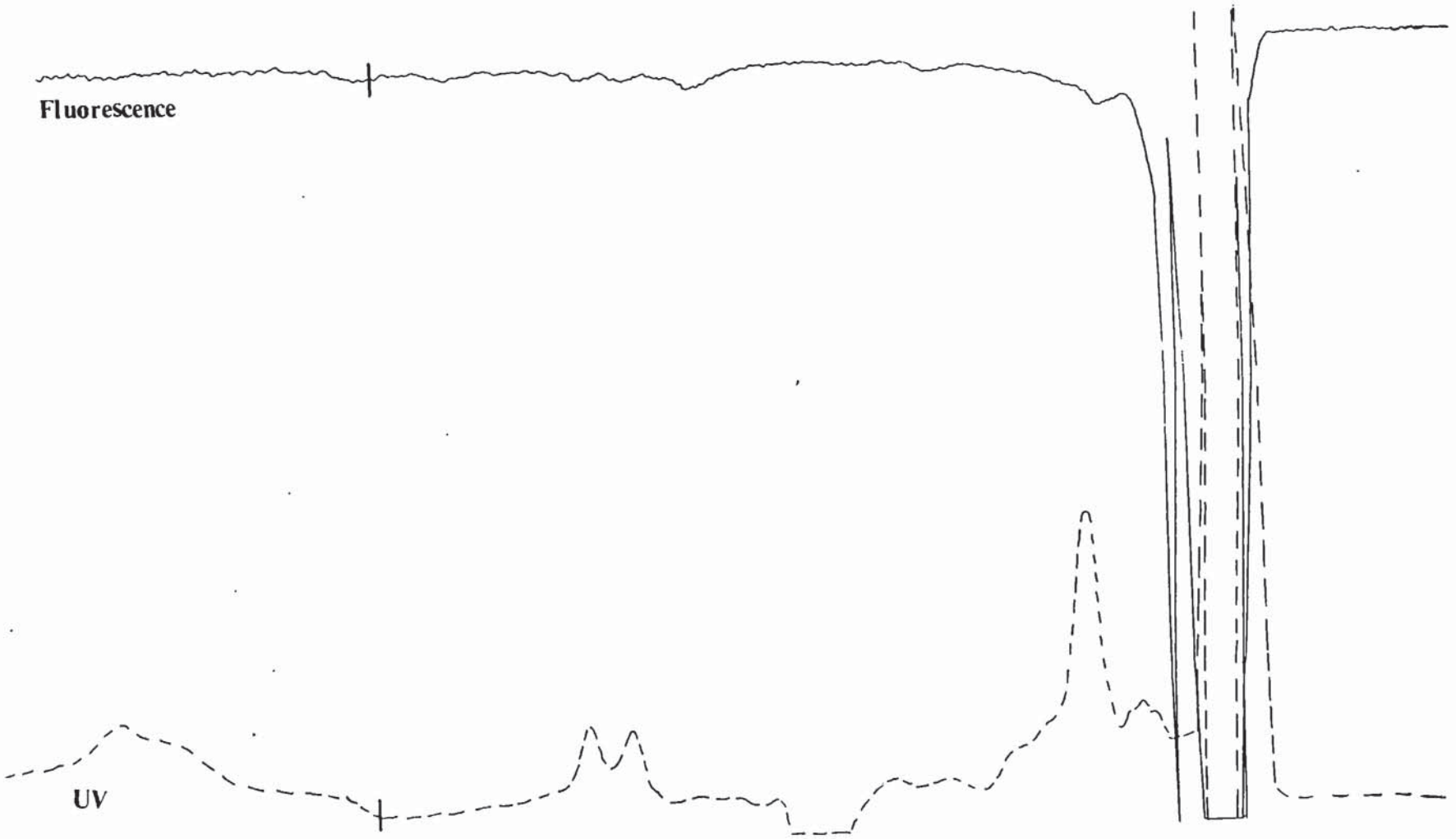


Figure 5.12:- Chromatogram of the excised white spot deposit from soft lens 1.

Figure 5.13:- Chromatogram of soft lens sample 2

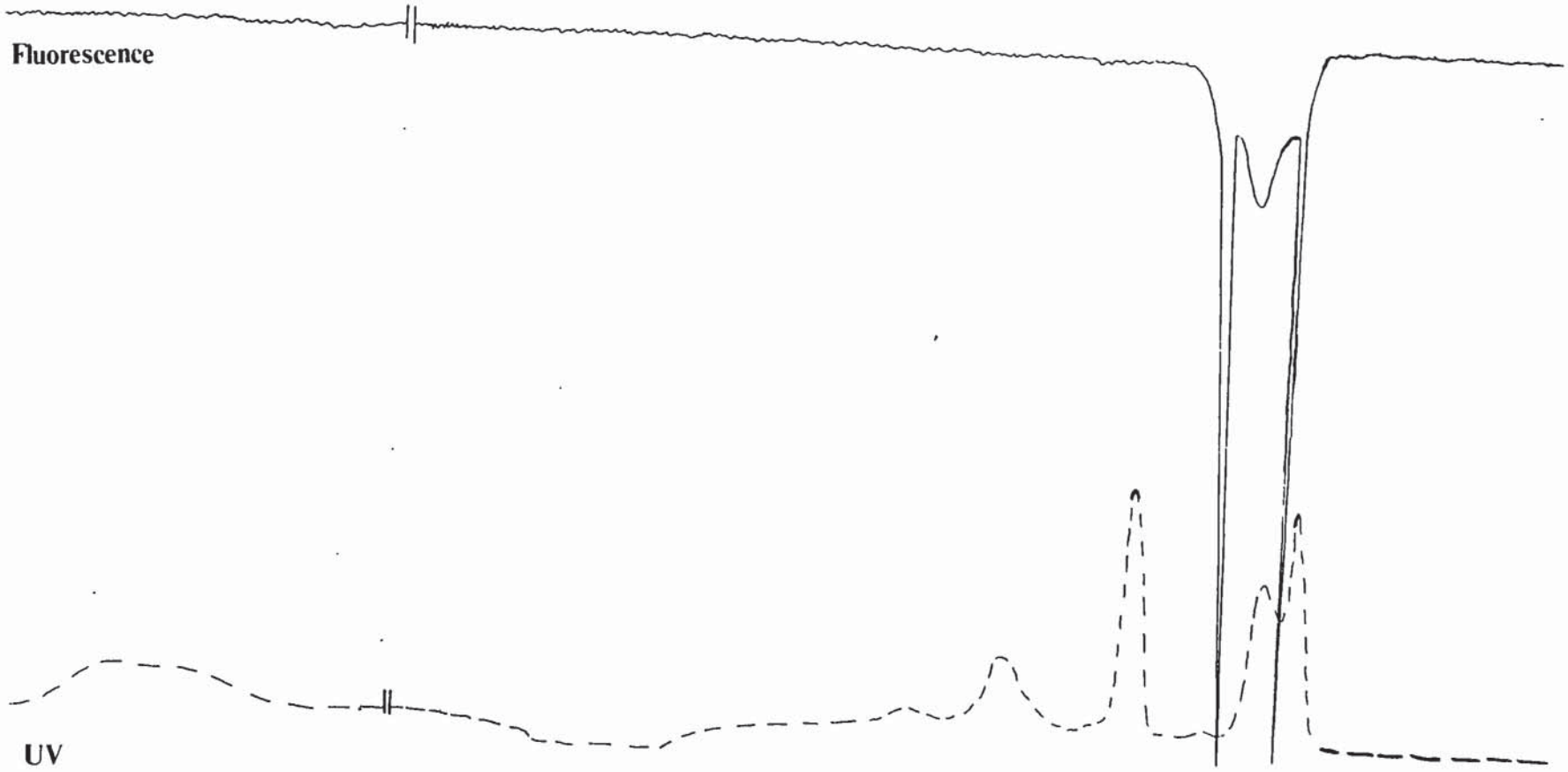


Figure 5.14:- Chromatogram of the excised white spot deposit from soft lens sample 2.

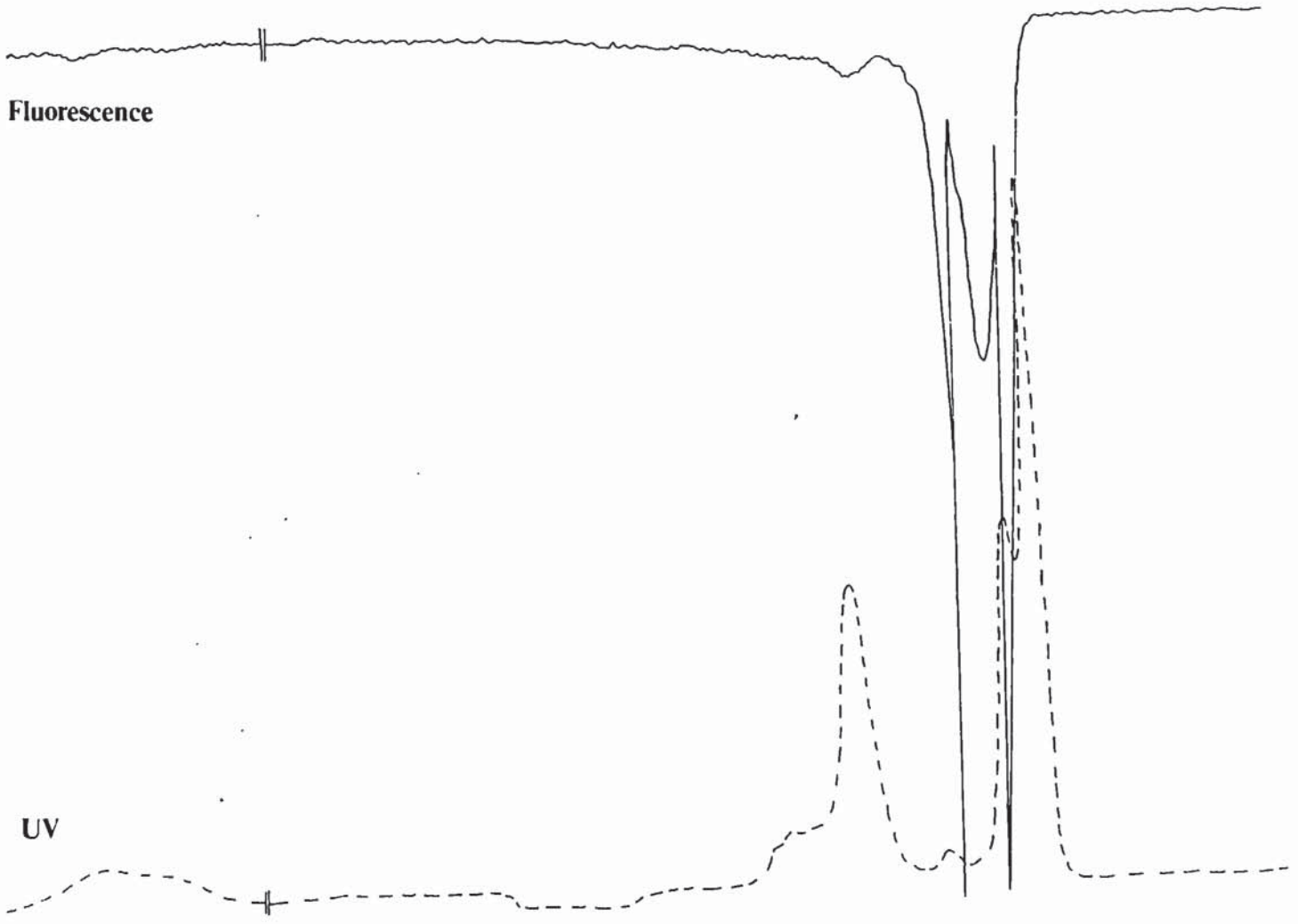


Figure 5.15:- Chromatogram of soft lens sample 3.

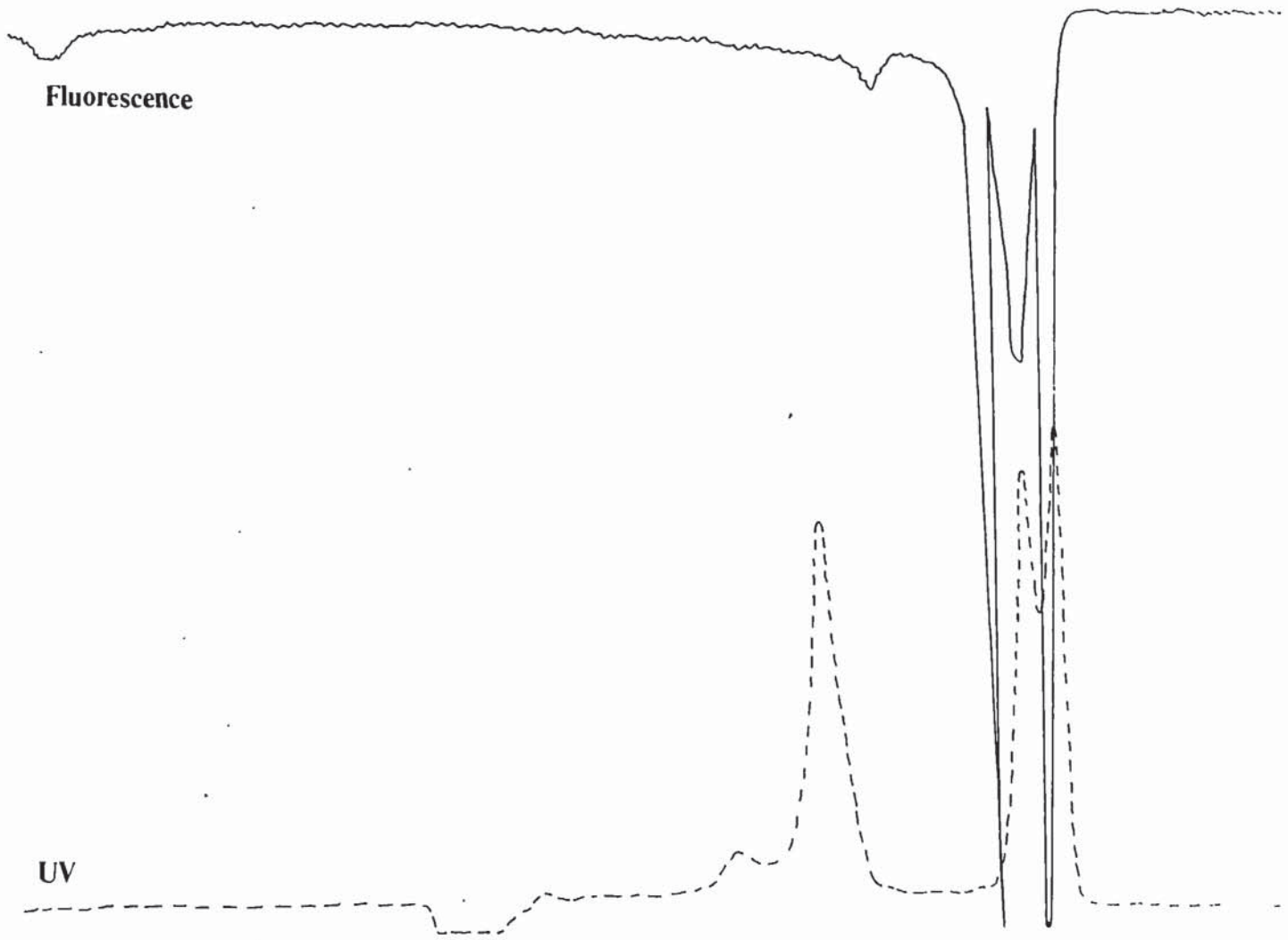


Figure 5.16:- Chromatogram of the excised white spot deposit from soft lens sample 3.

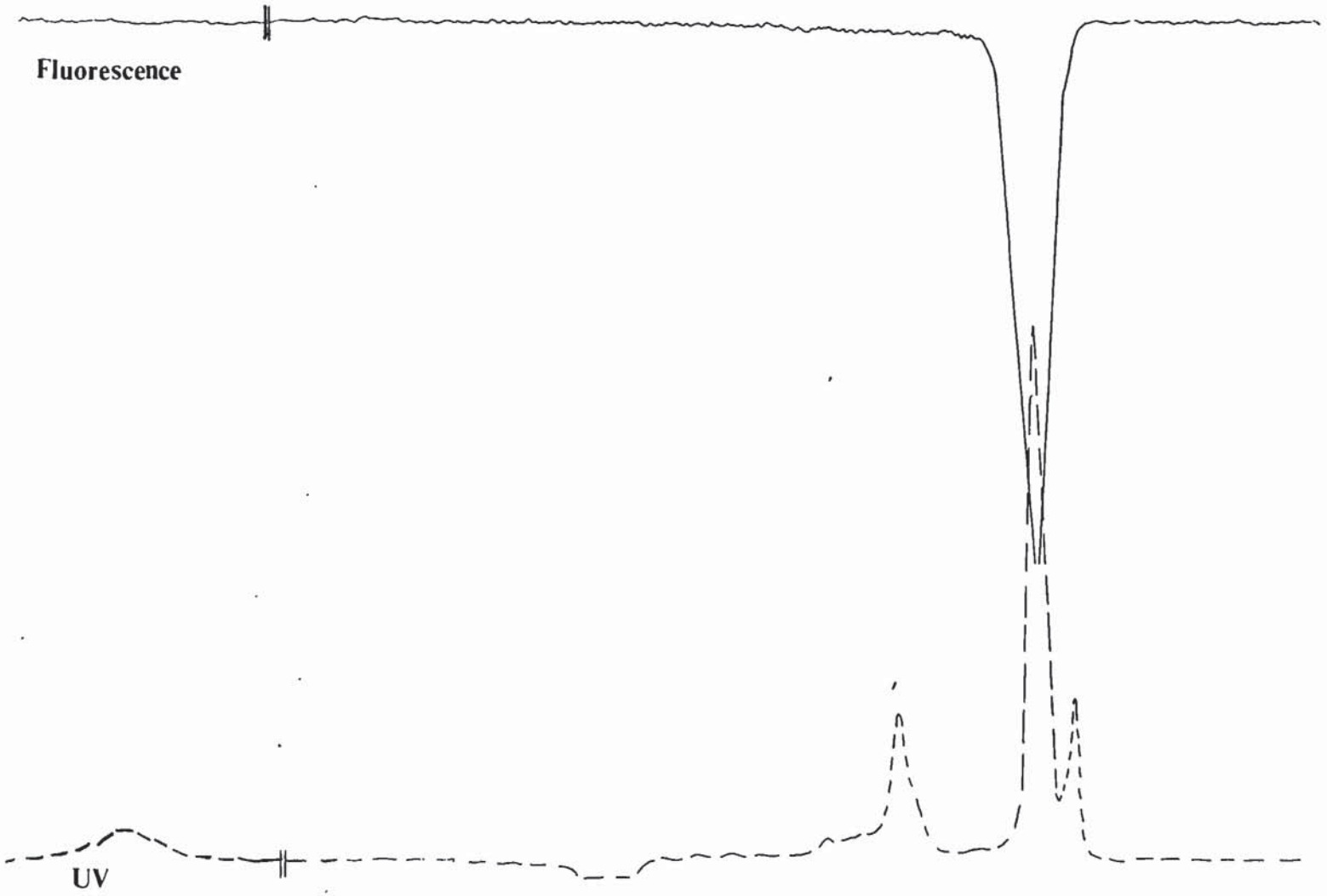


Figure 5.17:- Chromatogram of soft lens sample 4.

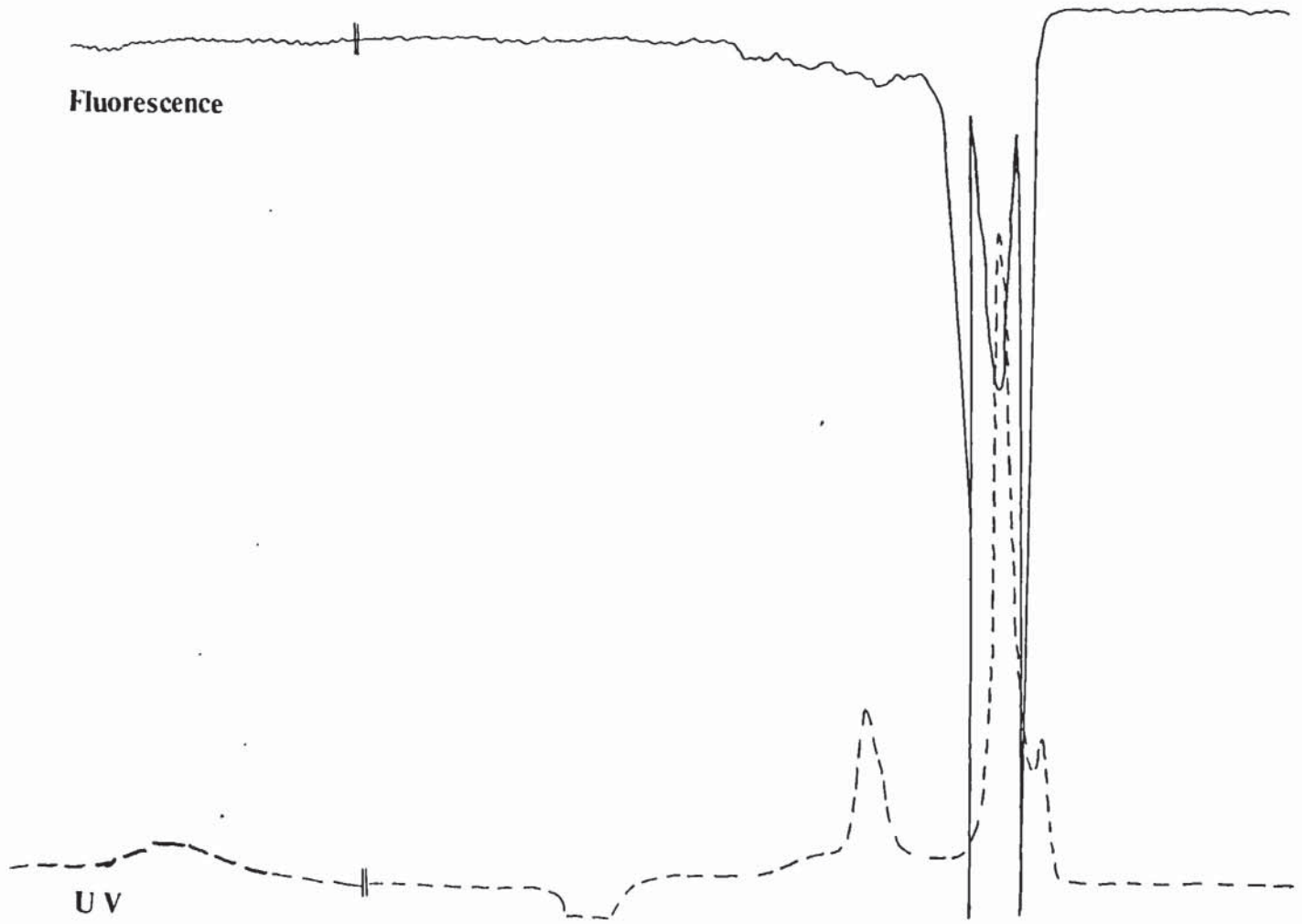
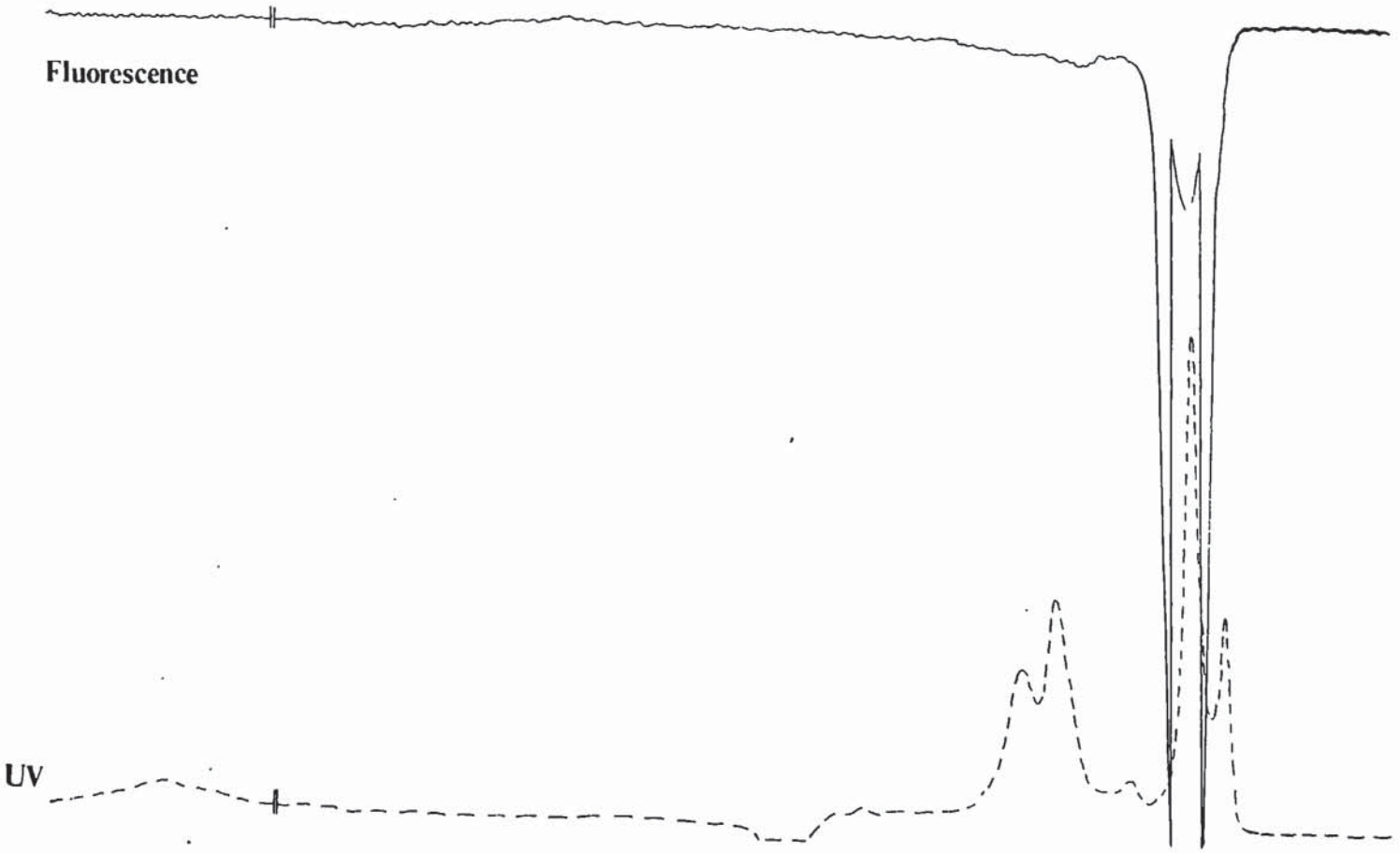


Figure 5.18:- Chromatogram of the excised white spot deposit from soft lens sample 4.



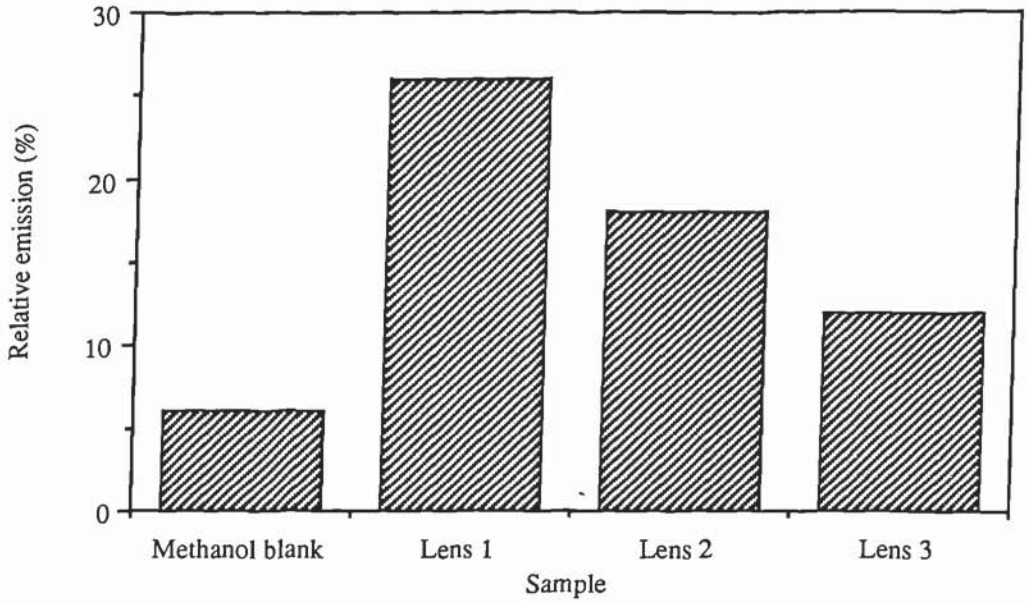
In addition to white spot deposits, calcium films are also observed on spoilt contact lenses (see chapter 3). The lipid species involved in these films were also investigated.

5.2.4. Calcium film deposits.

Calcium films are rough inorganic layers present on the majority of the lens surface which tend to be covered by a layer of organic material. A stained section of such a lens is shown in figure 3.29. The lipoidal component of the extractable organic layer was investigated. Three lenses with inorganic calcium films were analysed using fluorescence spectroscopy and high performance liquid chromatography (HPLC). The methanol extraction technique had no effect on the deposited inorganic layer present. It did however remove a layer of organic components from the surface of the lenses, as the methanol extract solution showed.

Preliminary observations had revealed that for all the lenses and methanol extracts the greatest fluorescence intensity of excitation occurred at 360nm. As a result the emission spectra were all run at this excitation wavelength. For all three lenses an increase in the fluorescence intensity value around 400-550 nm occurs with the methanol extracts, which is indicative of lipoidal material being extracted from the surface of the contact lenses. The methanol extracts from these lenses were then analysed by high performance liquid chromatography.

Figure 5.19:- Fluorescence intensity values of the calcium film lenses after extraction and the methanol extracts.

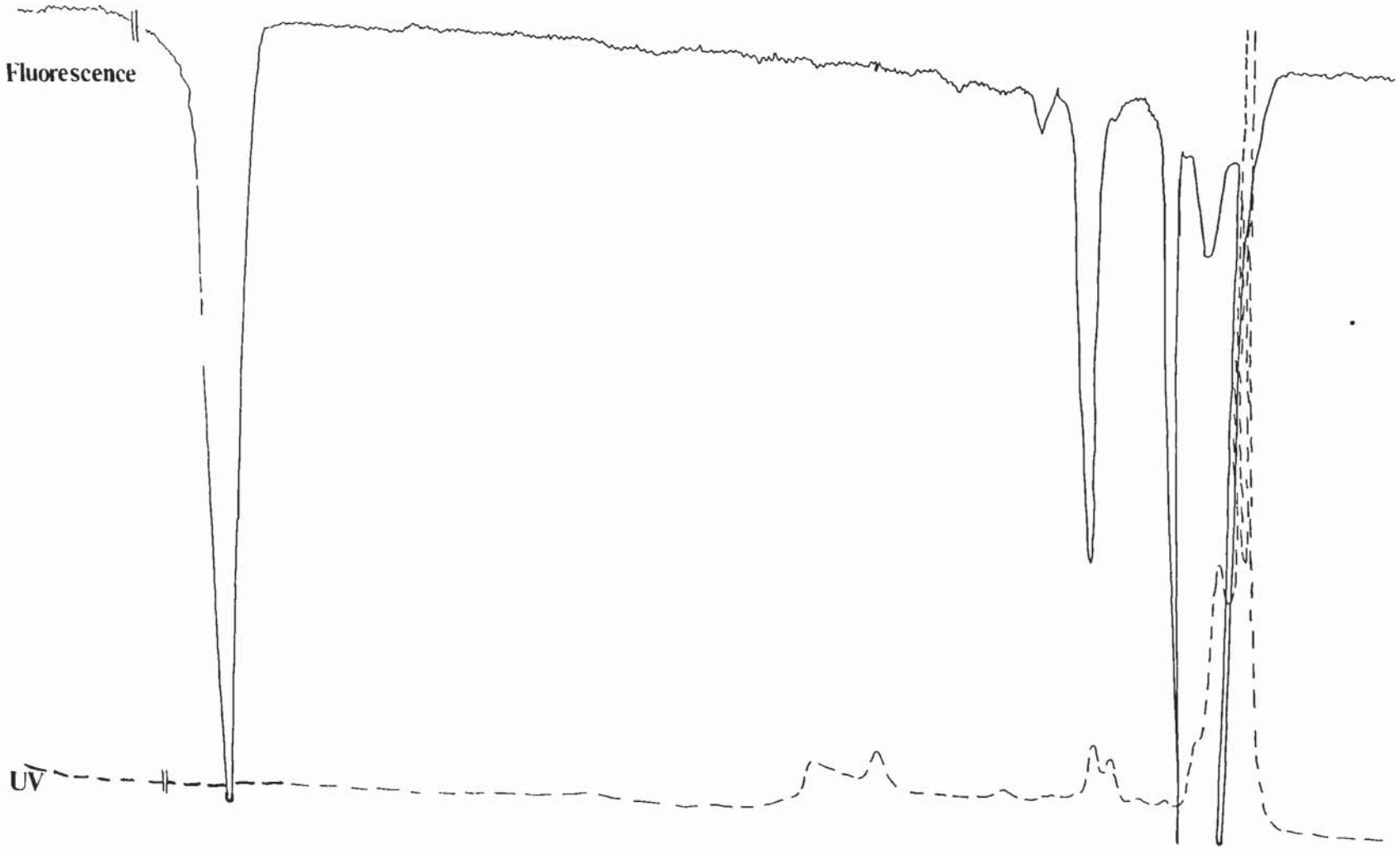


All three lenses show cholesterol ester and fatty acid components. Lenses 151 (figure 5.20) and 154 (figure 5.21) also have a cholesterol component, whereas lens 156 (figure 5.22) has a triglyceride component. The predominant feature of the HPLC traces derived from these lenses was the high proportion of cholesterol esters with low levels of fatty acids plus triglycerides (lenses 151 and 154) and cholesterol (lens 156). This may be due to some stimulation of the Meibomian glands by the undulating surface of the calcium film. The total lipid peak areas of the calcium film lenses were similar to each other.

Table 5.5:- Retention times of the methanol extracts of the calcium film lenses.

	Rt	Possible identity		Rt	Possible identity
151	71	Cholesterol ester	**154**	65	Cholesterol ester
	79	Cholesterol ester		73	Cholesterol ester
				85	Cholesterol ester
	190	Fatty acid		198	Fatty acid
	1140	Cholesterol		1000	Cholesterol
156	62	Cholesterol ester			
	72	Cholesterol ester			
	110	Triglyceride			
	141	Triglyceride			
	217	Fatty acid			

Figure 5.20:- Chromatogram of calcium film lens 151.



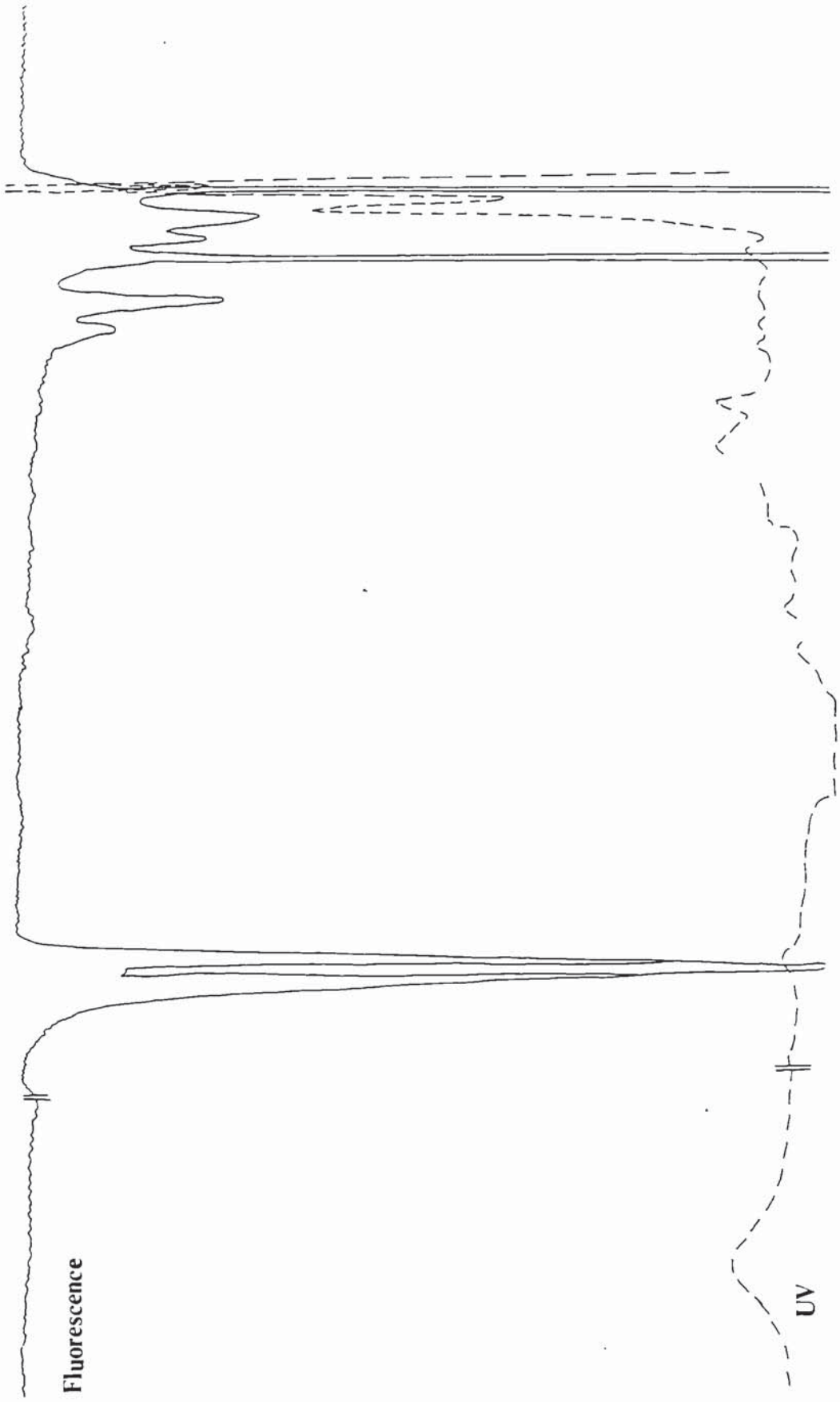
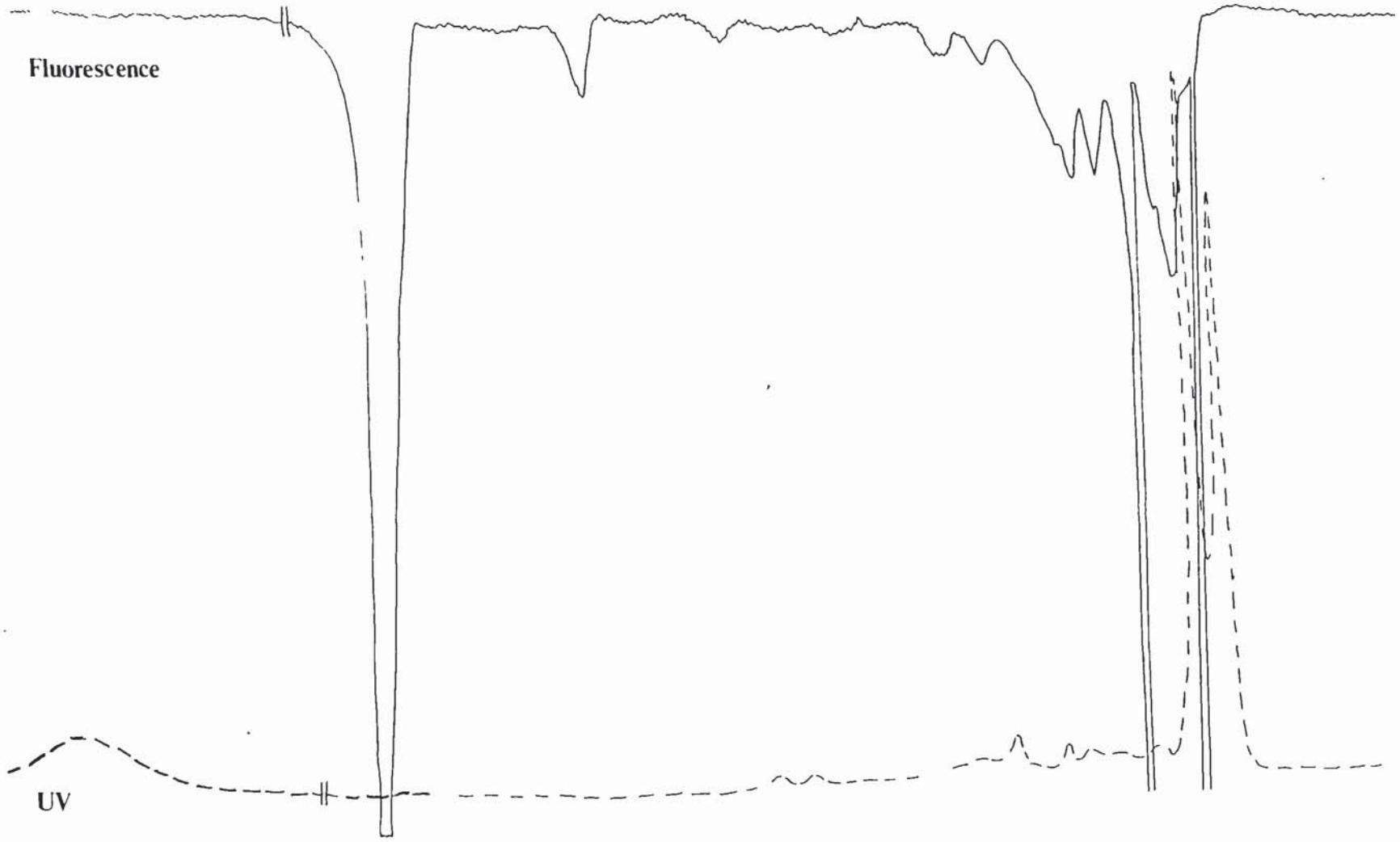


Figure 5.21:- Chromatogram of calcium film lens 154.

Figure 5.22:- Chromatogram of calcium film lens 156.



5.2.5. Analysis of hard (Rigid gas permeable) contact lenses (Boston II).

The pattern of spoilation on the hard lenses was studied in order to attempt to establish whether the techniques were sensitive enough and applicable to hard lenses and whether any differences occur between the lipoidal deposition on hard and soft lens compositions.

The two pairs of Boston hard contact lenses (siloxy-methacrylate-itaconate copolymer) were analysed. One pair had been worn daily for 22 months and the second pair for 18 months. All the lenses were extracted separately using 3ml of methanol for 30 minutes on a shaker.

The Boston lenses worn daily for 22 months show similar chromatograms (figure 5.23, figure 5.24) for both eyes. Both lenses show peaks with retention times indicative of cholesterol esters and triglycerides. There is also probably diglyceride and phospholipid present as the retention times of these groups are masked by the other classes of lipid present. The right eye has a cholesterol component. The pattern of peak areas observed is triglyceride, cholesterol esters, cholesterol and other lipid classes. The total lipid peak areas are similar for each eye.

Table 5.6:- Boston hard contact lenses worn daily for 22 months.

RIGHT		LEFT	
Rt	**Possible identity**	**Rt**	**Possible identity**
67	Cholesterol ester	65	Cholesterol ester
80	Cholesterol ester	73	Cholesterol ester
85	Cholesterol ester	82	Cholesterol ester
105	Triglyceride	102	Triglyceride
116	Triglyceride	111	Triglyceride
133	Triglyceride	132	Triglyceride
144	Triglyceride	146	Triglyceride
825	Cholesterol	653	

Figure 5.23:- Chromatogram of a Boston hard lens worn daily for 22 months in the right eye.

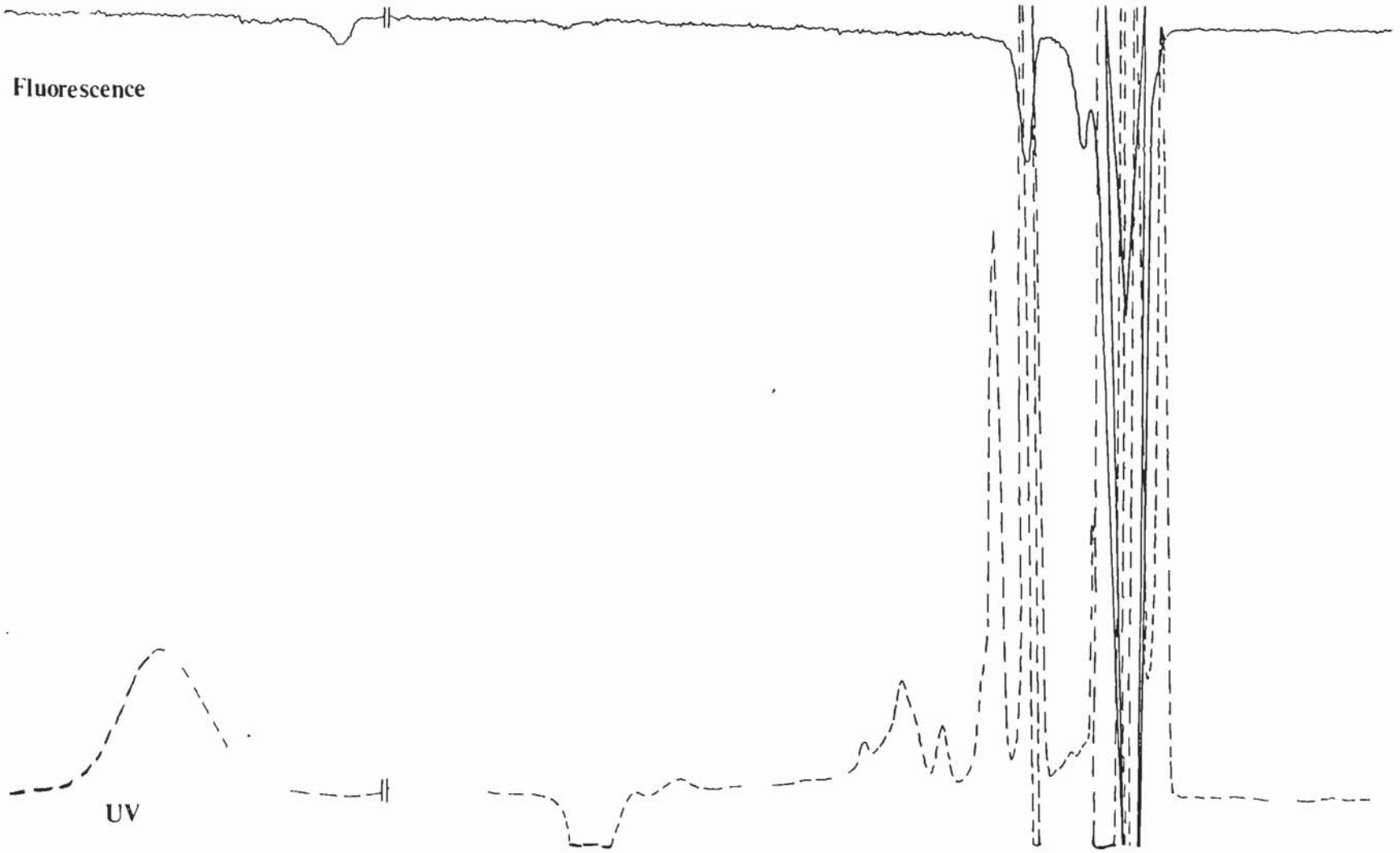
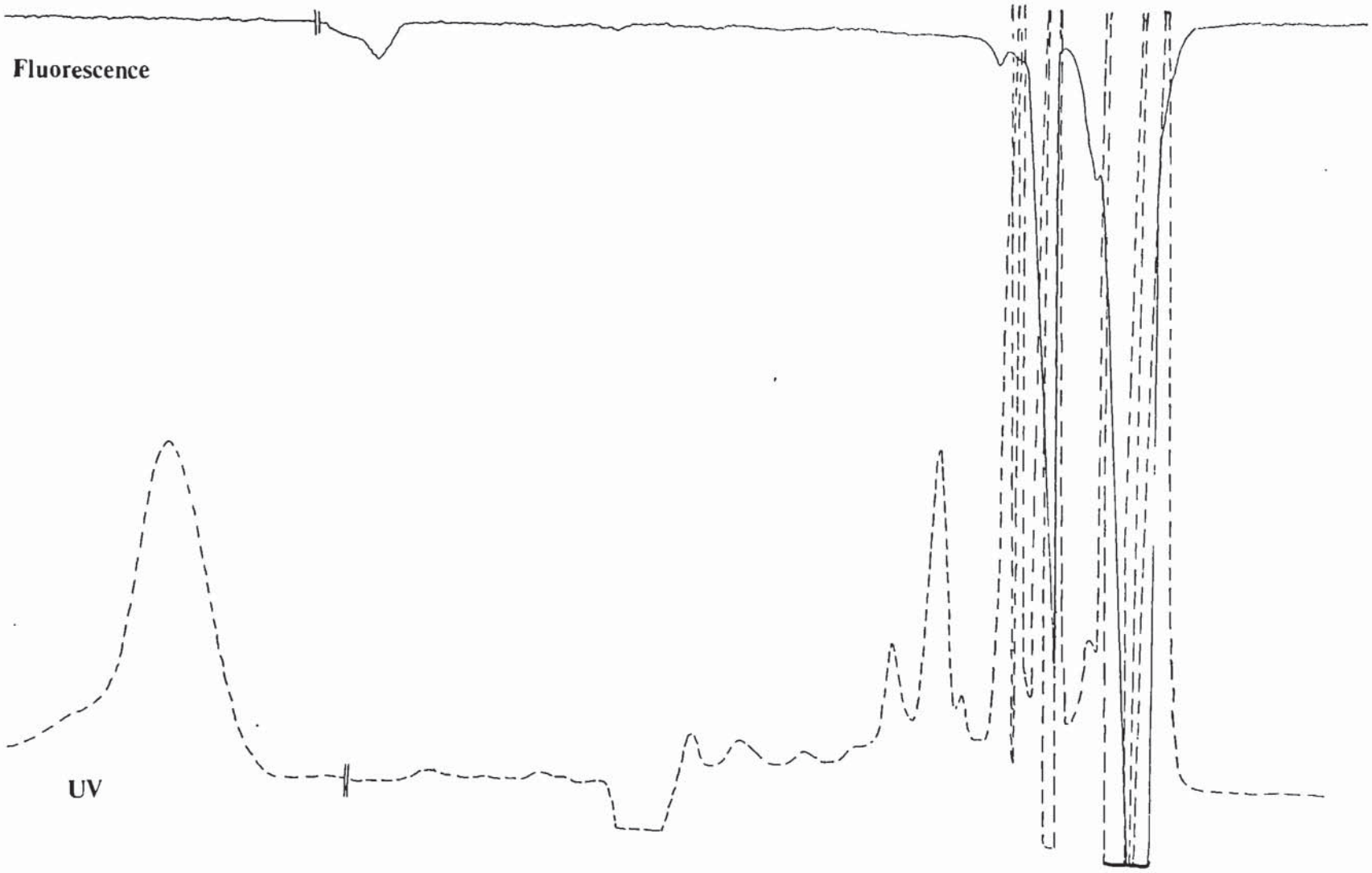


Figure 5.24:- Chromatogram of a Boston hard lens worn daily for 22 months in the left eye.



The Boston lenses worn daily for 18 months also exhibit comparable patterns of retention times for the left and right eyes (figures 5.25, 5.26). The retention times are indicative of cholesterol esters, triglycerides, fatty acids and probably diglyceride and phospholipid. The right eye also has a cholesterol component. This pair of lenses also show a similar total lipid peak area. This is however lower than the 15 minute tear envelope result obtained from the same patient. The eyes show a difference in lipid peak area pattern. For the right eye the peak areas decrease cholesterol esters, triglycerides, cholesterol, fatty acids and other lipid classes and for the left eye the pattern is triglyceride, cholesterol esters and fatty acids.

Table 5.7:- Boston hard contact lenses worn daily for 18 months.

RIGHT		LEFT	
Rt	**Possible identity**	**Rt**	**Possible identity**
67	Cholesterol ester	66	Cholesterol ester
80	Cholesterol ester	78	Cholesterol ester
99	Triglyceride	94	Triglyceride
103	Triglyceride	135	Triglyceride
124	Triglyceride	147	Triglyceride
198	Fatty acid	220	Fatty acid
525			
888	Cholesterol		

Figure 5.25:- Chromatogram of a Boston hard lens worn daily for 18 months in the left eye.

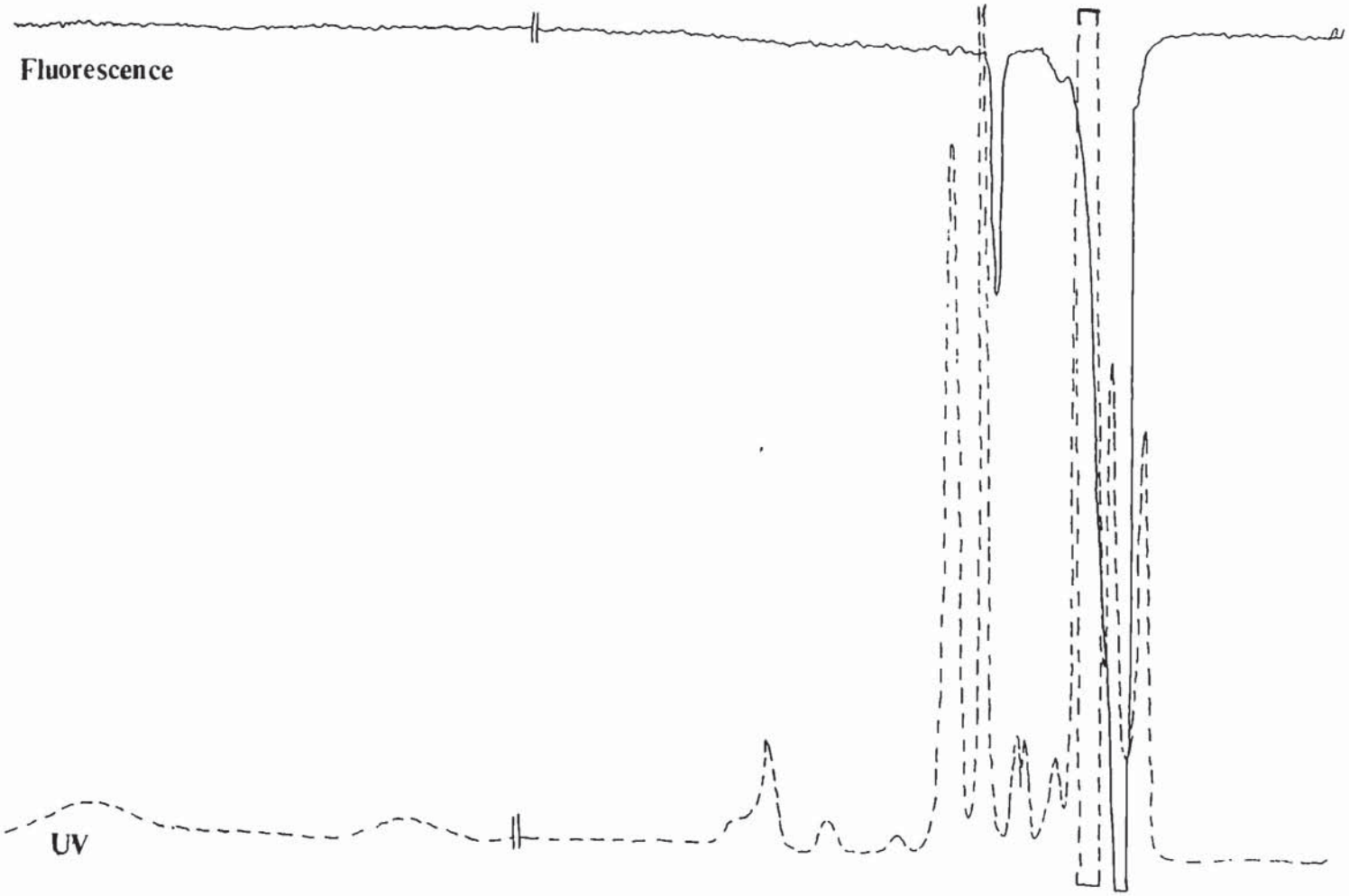
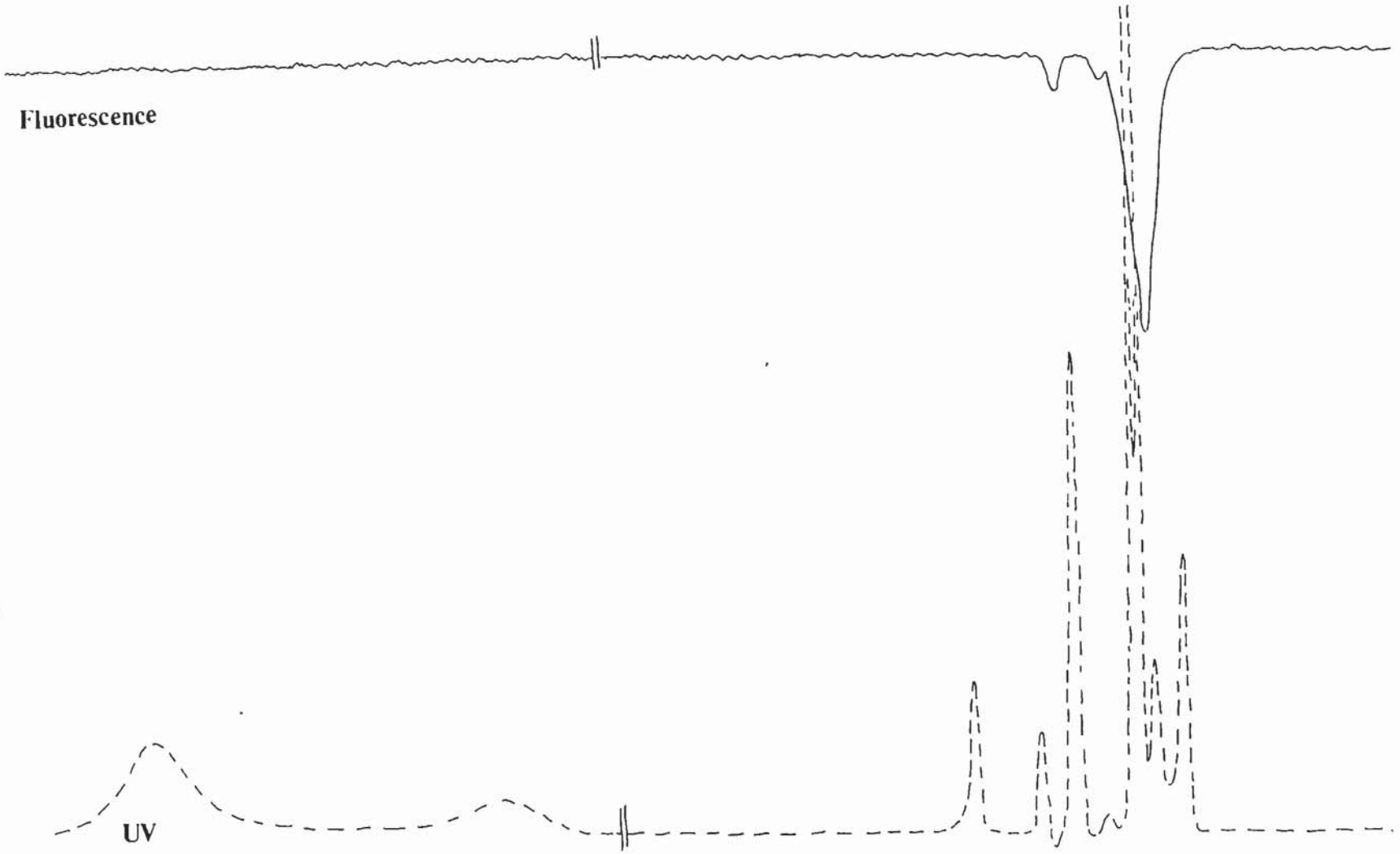


Figure 5.26:- Chromatogram of a Boston hard lens worn daily for 18 months in the right eye.



In addition to analysing the lipid species present in the spoilation phenomena and tear film. Lipids may also be transferred onto the contact lens from extrinsic sources. One such extrinsic factor was investigated.

5.2.6. Sterile handled lenses.

Extrinsic factors are known to be involved in the deposition process [10]. During this study all the contact lenses have been handled using clean forceps and gloves. Lipids occur naturally on the skin surface along with other components which are excreted through the skin, like sodium chloride, other ions, drugs etc. [35]. The lipids are fairly similar in composition what ever their source of production. It is therefore likely that components are deposited onto the contact lenses during handling. In order to determine whether this is the case and the quantity of lipids deposited onto the lenses, two studies have been performed.

In the initial study a sterile lens was handled, after washing the hands, for 5 minutes prior to methanol extraction. Fluorescence spectra were run before and after lens handling. In addition to this test the extractable lipids present on a finger were determined after hand washing, to compare and contrast them with the lipids deposited onto the sterile contact lens. The fluorescence spectra of the sterile handled lens and lipoidal species extracted from the thumb were run at 360nm.

Table 5.8:- Fluorescence spectra results

Excitation peak	Spoilation peak	Secondary excitation peak
Lens, pre-extraction		
360-410 (+100)	410-510 (7)	700-770 (20)
Methanol extract		
360-410 (+100)	410-510 (10)	700-770 (9)
Lens, post-extraction		
360-410 (+100)	410-510 (8)	700-770 (16)

The total lipid peak area for the extract of the skin lipids from the thumb (figure 5.28) was much greater than those extracted from the sterile handled lens (figure 5.29). The number of lipid classes present in the thumb extract was also greater. The lens peak areas decrease in the order cholesterol esters, triglycerides and other lipid classes as opposed to triglycerides, cholesterol esters, fatty acids and other lipid classes observed with the thumb extract.

Table 5.9:- Retention times of extracted lipids.

	Rt	Possible identity			
Lens	66	Cholesterol ester	**Thumb**	65	Cholesterol ester
	73	Cholesterol ester		67	Cholesterol ester
	111	Triglyceride		111	Triglyceride
				124	Triglyceride
				154	Triglyceride
				169	Fatty acid
	380			276	Fatty acid
	662	Fatty alcohol		631	Fatty alcohol

Figure 5.27:- Chromatogram of a sterile lens extracted after handling for five minutes.

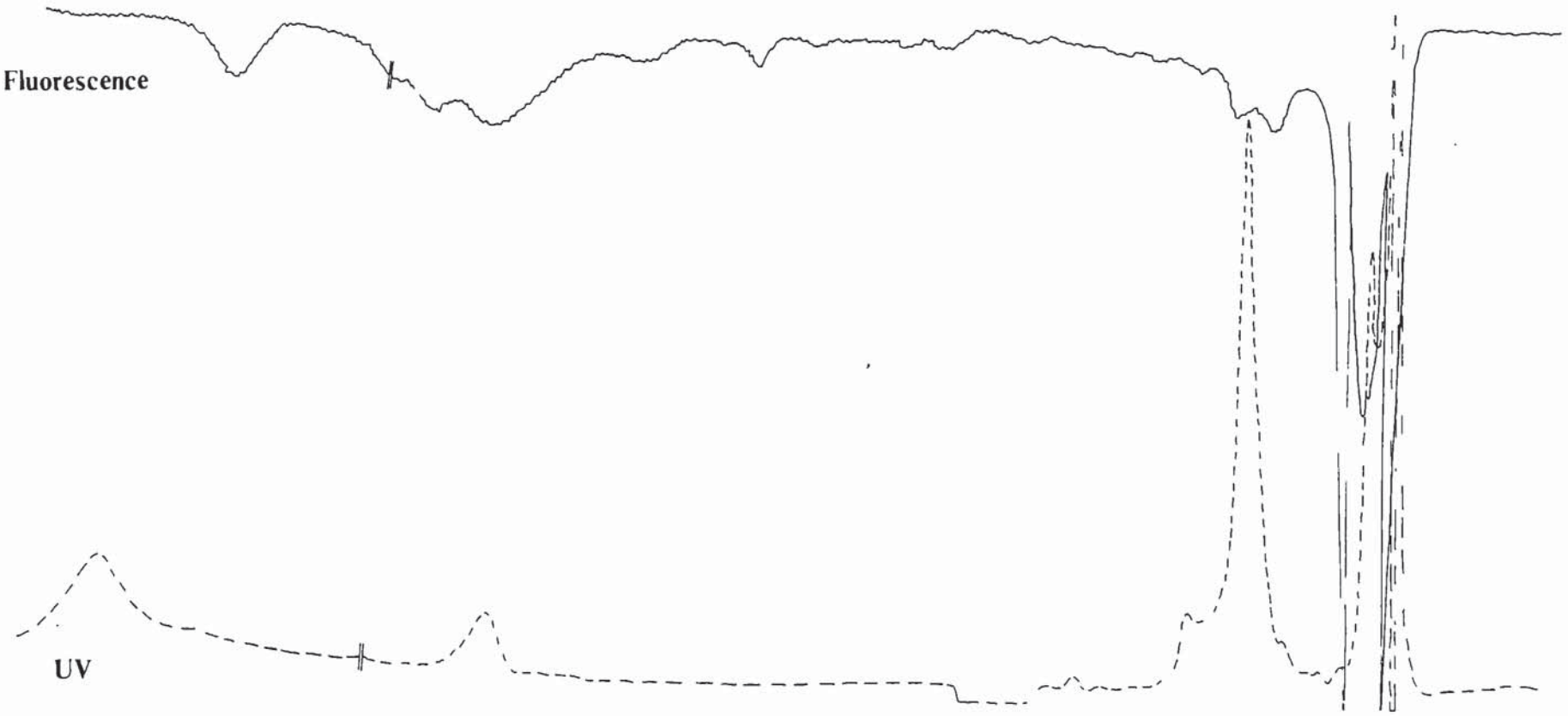
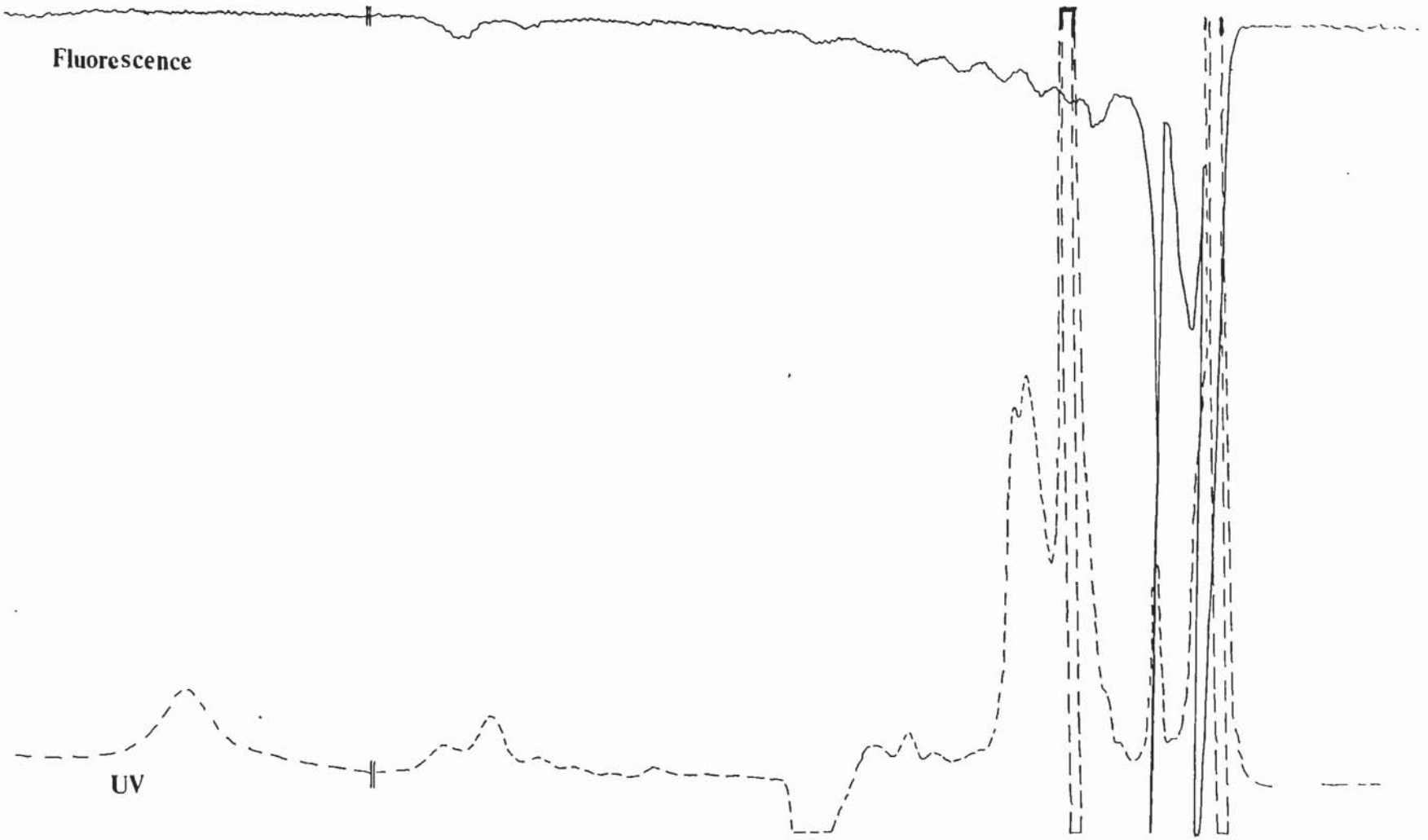
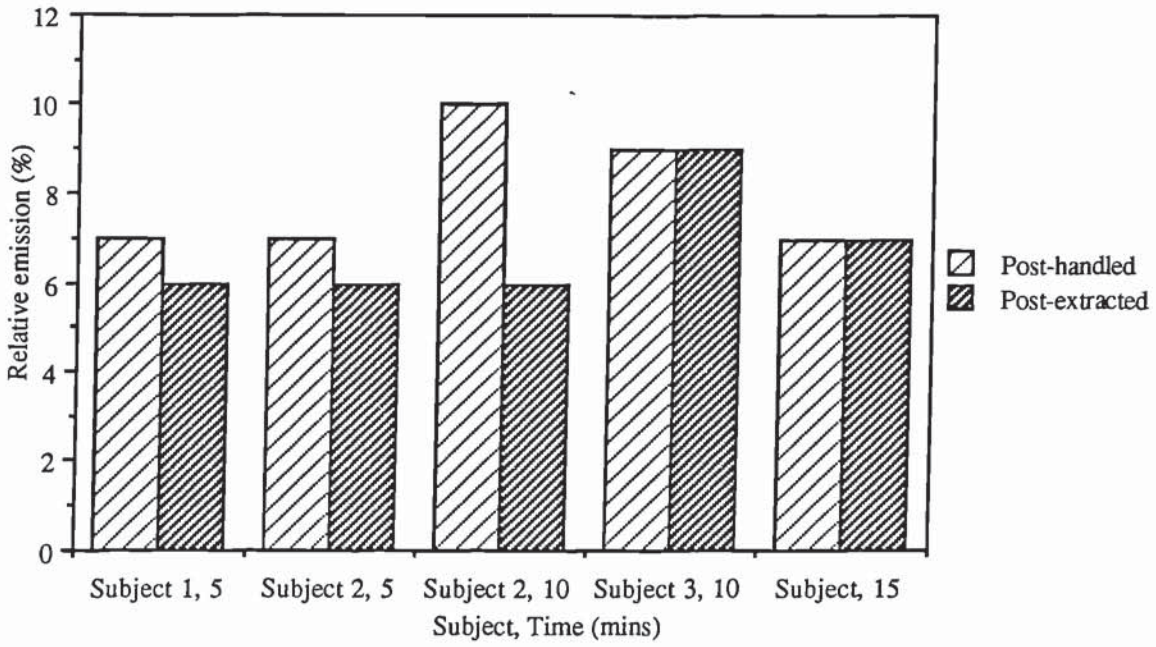


Figure 5.28:- Chromatogram of a the lipids extracted from the skin after five minutes.



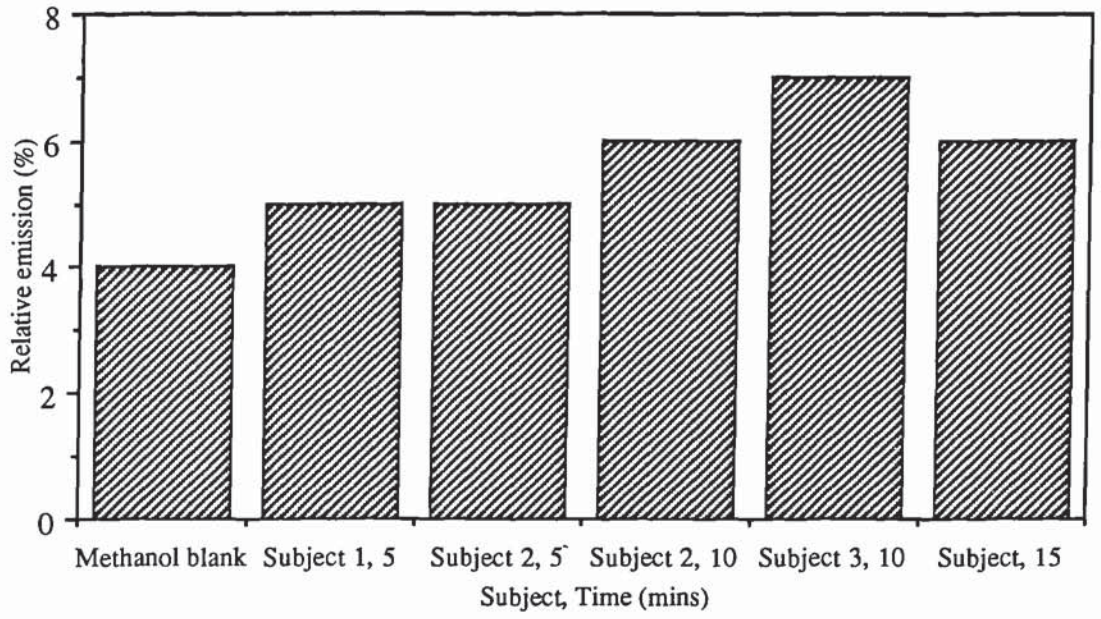
This preliminary study showed that lipids were deposited onto the surface of a sterile contact lens during handling. Thus it was decided to test the effect of the length of time of handling and variation between patients sebum lipids. The lenses were handled for 5, 10 and 15 minutes prior to extraction and analysis. The fluorescence spectra of the sterile handled lenses were run at 360nm.

Figure 5.29:- Fluorescence intensity results for the sterile handled lenses.



These fluorescence results indicate that biological material is deposited onto the contact lens. The emission intensity after 5 and 10 minutes are similar for each subject tested. There was however a slight decrease in the quantity of biological material deposited onto the lens after 15 minutes with subject 3.

Figure 5.30:- Fluorescence intensity values of the methanol extracts of the sterile handled lenses.



The quantity of biological material extracted into the methanol was fairly similar for all three subjects over all time periods as observed using the fluorescence spectrophotofluorimeter.

Table 5.10:- Retention times of extractable lipids from the contact lenses.

Subject	Rt	Possible identity
1, 5min.	68	Cholesterol ester
	78	Cholesterol ester
	142	Triglyceride
	255	Fatty acid
	706	Cholesterol
2, 5min.	68	Cholesterol ester
	77	Cholesterol ester
	122	Triglyceride
	182	Fatty acid
	663	
2, 10min.	69	Cholesterol ester
	76	Cholesterol ester
	117	Triglyceride
	126	Triglyceride
	130	Triglyceride
	172	Fatty acid
	249	Fatty acid
	659	

Table 5.10:-(continued) Retention times of extractable lipids from the contact lenses.

3, 10min.	70	Cholesterol ester
	80	Cholesterol ester
	127	Triglyceride
	719	Cholesterol
3, 15min.	71	Cholesterol ester
	77	Cholesterol ester
	112	Triglyceride
	121	Triglyceride
	161	Triglyceride
	601	

The extracted lenses from two subjects have the following lipid classes; cholesterol esters, triglycerides, fatty acids and other lipid classes. The third subject only had cholesterol esters, triglycerides, cholesterol and other lipid classes. In all cases a fatty alcohol or monoglyceride component was extracted with a retention time of 601-664 seconds.

Figure 5.31:- Chromatogram of a sterile lens extracted after handling for five minutes by subject 1.

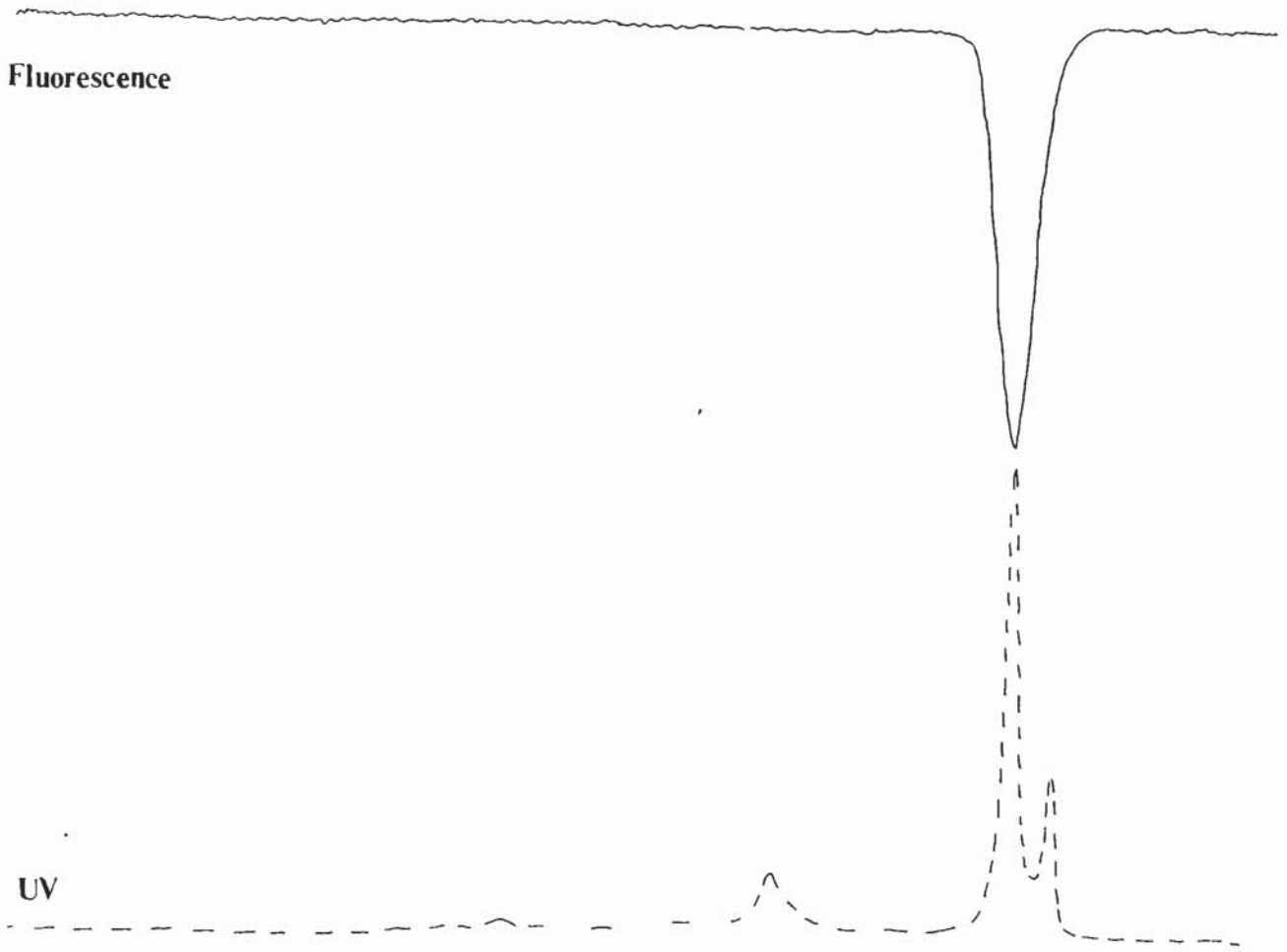


Figure 5.32:- Chromatogram of a sterile lens extracted after handling for five minutes by subject 2.

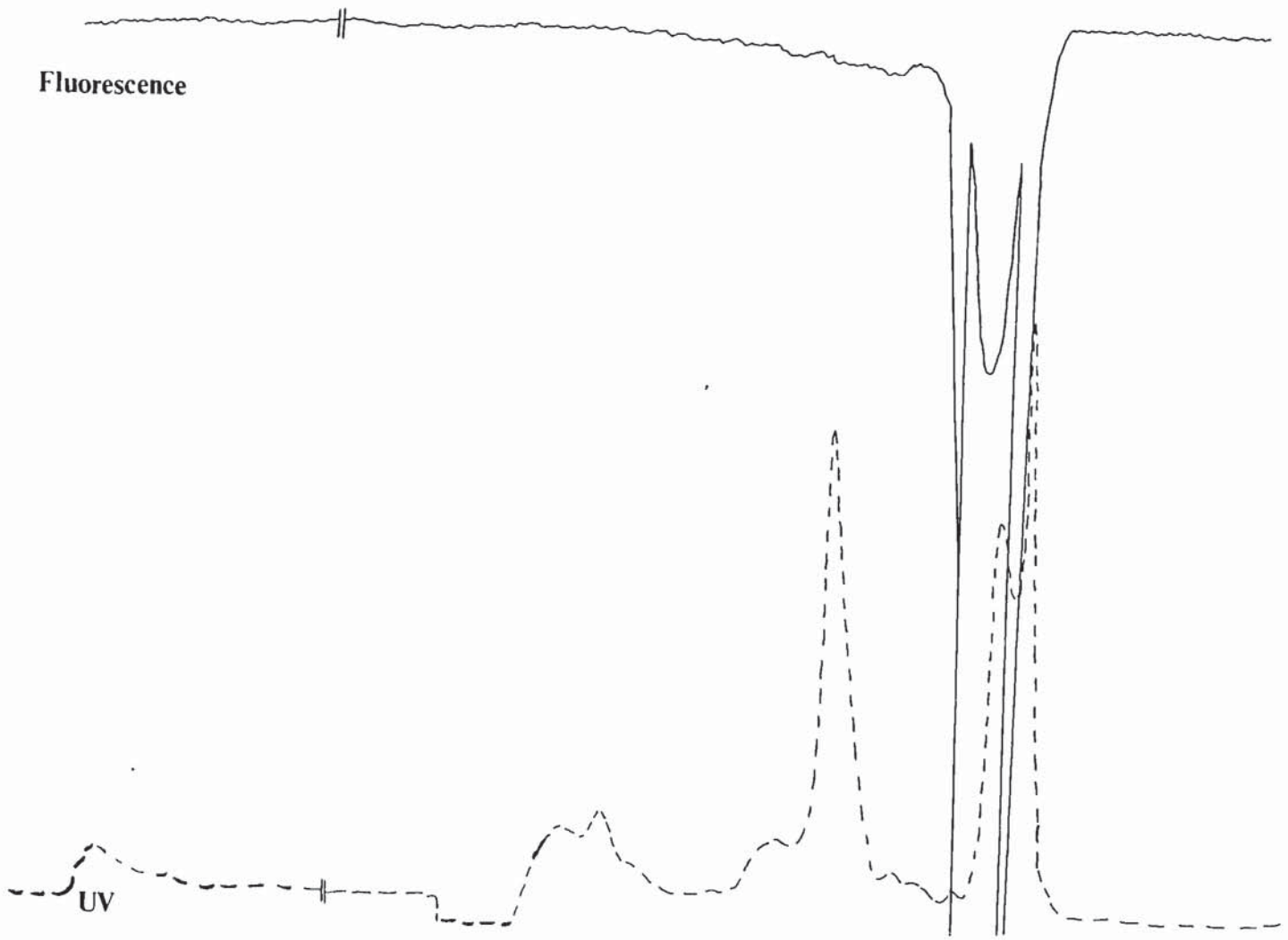


Figure 5.33:- Chromatogram of a sterile lens extracted after handling for ten minutes by subject 2.

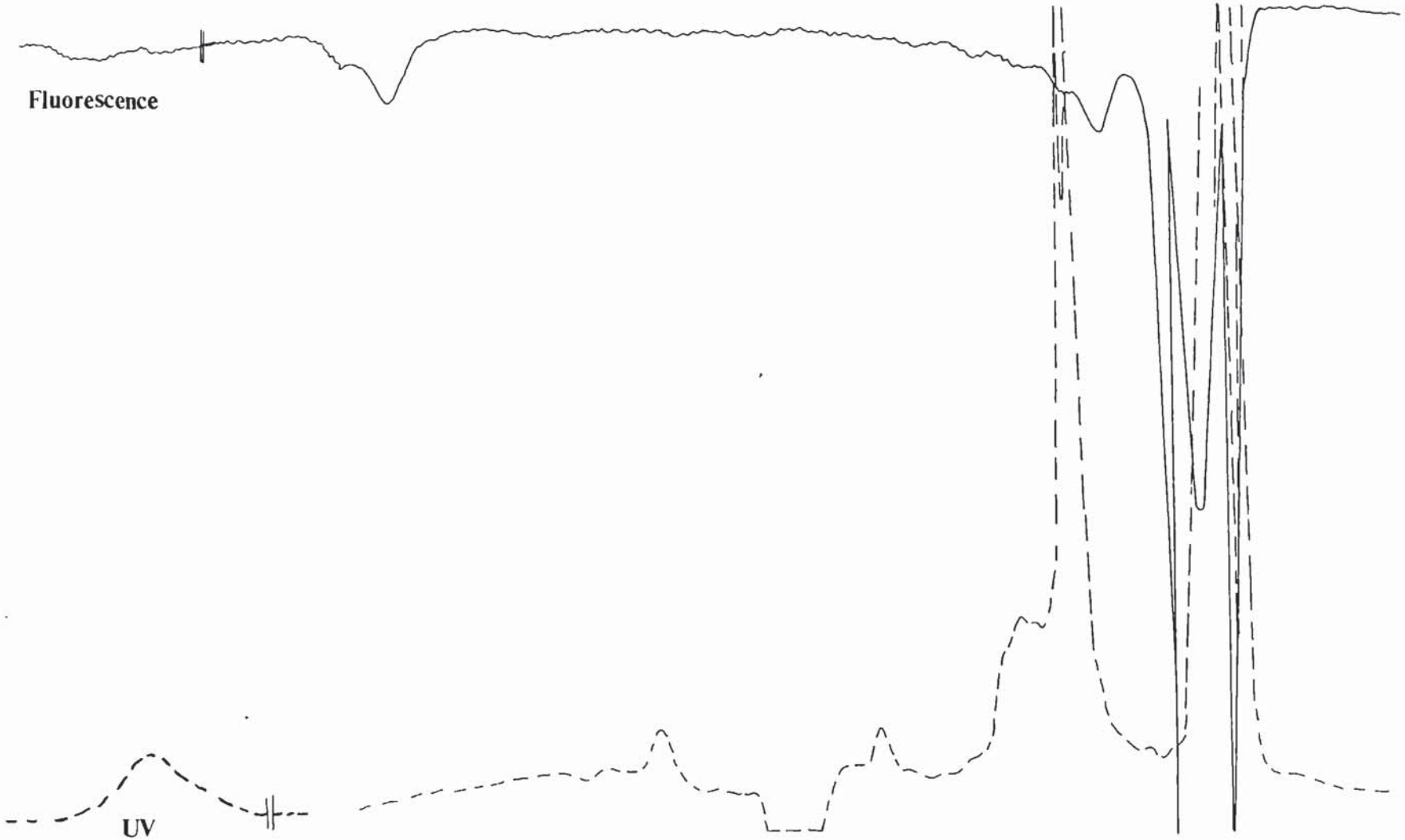


Figure 5.34:- Chromatogram of a sterile lens extracted after handling for ten minutes by subject 3.

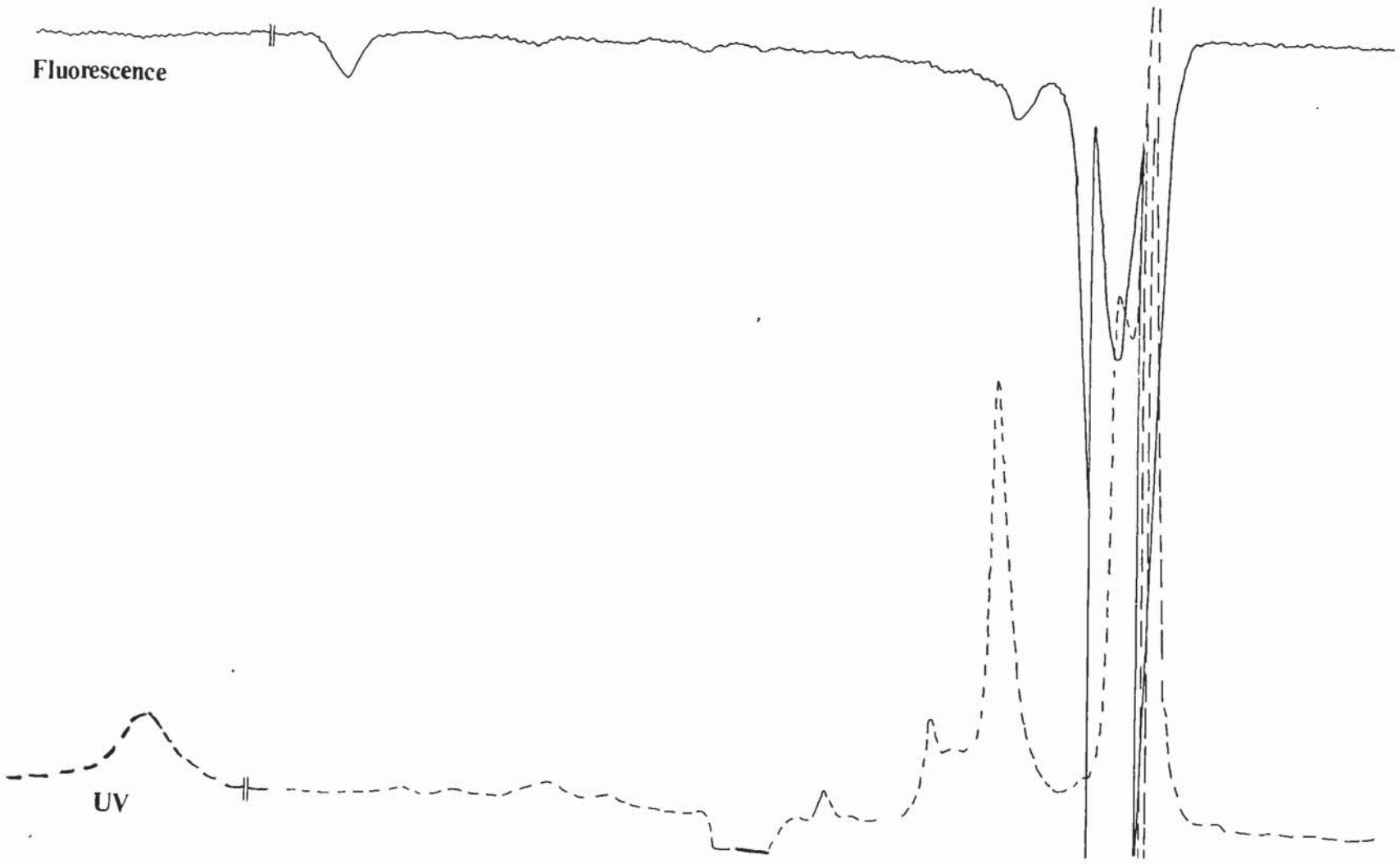
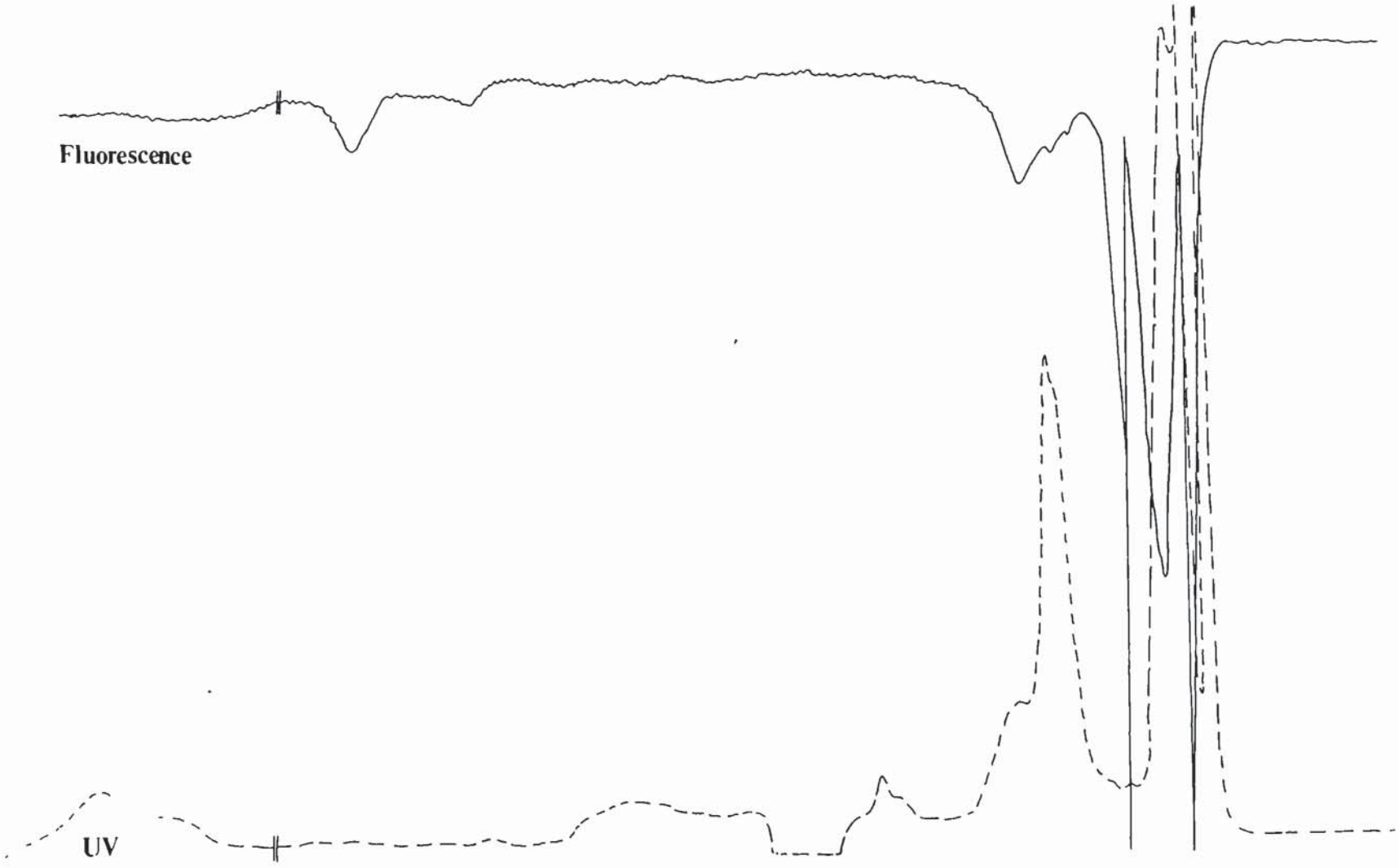


Figure 5.35:- Chromatogram of a sterile lens extracted after handling for fifteen minutes by subject 3.



The lipids found in human surface lipid are comparable to tears containing free fatty acids, wax and cholesterol esters, triglycerides, sterols, diglycerides, monoglycerides phospholipids and squalene [234-236]. Squalene and paraffins are not present in tears. The paraffins are usually due to extrinsic factors such as cosmetics, floor wax, furniture polish, wax cartons, oil etc.. Any components less or equally polar to the cholesterol esters are eluted in the primary peak using this solvent system. This would include the hydrocarbons such as squalene and carotene and the simple esters such as the wax esters. These classes could be separated further by using normal phase high performance liquid chromatography and a less polar solvent system or by using reversed phase columns.

The approximate concentrations of the lipid deposited onto the lens varied between patients. Subjects 1 and 2 had similar peak areas after 5 minutes whereas subject 3's peak area was approximately double the area of the former two's. After a further 5 minutes the peak areas for subjects 2 and 3 had increased by about half again and after a further 5 minutes by approximately a third again for subject 3. The thumb extract had the highest peak area indicating only a proportion of the skin lipid is transferred to the lens during handling.

These results indicate that a variable amount of lipid can be transferred onto the contact lens during handling. The increase in peak area occurs after 5 minutes so the amount transferred will depend upon the length of time for which the lenses are handled. These lipids may however contribute to the ocular spoilation process however as there are strong similarities between the lipid secretions present and the other components present in tears.

5.3. Summary and conclusions from chapter 5.

These studies show that it is possible to analyse the minute quantities of lipoidal material deposited onto soft and hard contact lenses after a very short period of time as well as analysing the products of spoilation. The mobile phase used separates components on the basis of the polarity of the functional group. This produces separation of the lipid classes in particular the triglycerides, cholesterol esters, fatty acids and cholesterol. Some overlap does however occur with these classes at low concentrations and also with diglyceride, monoglyceride, fatty alcohols and phospholipids. This method does however give a good general profile of the patients' tear chemistry and allow the relative proportions of the lipid classes to be assessed. It is also possible to produce a pattern of lipids similar to those obtained by lens extraction using standard lipid solutions figures 5.36, 5.37, 5.38. These standard lipid mixture chromatograms showed less overlap between classes, which may be due to the smaller number of lipids present in the solution.

At this stage it was felt important to establish the validity of the technique with a range of sample (i.e. worn lens) types. No attempt has been made to correlate detailed differences, or the development of specific chemical features in the deposited layer. To do this will require careful control of patients and their wear protocol and careful isolation of lens samples at appropriate stages in the wear programme. Some general conclusions can, however, be made from this study.

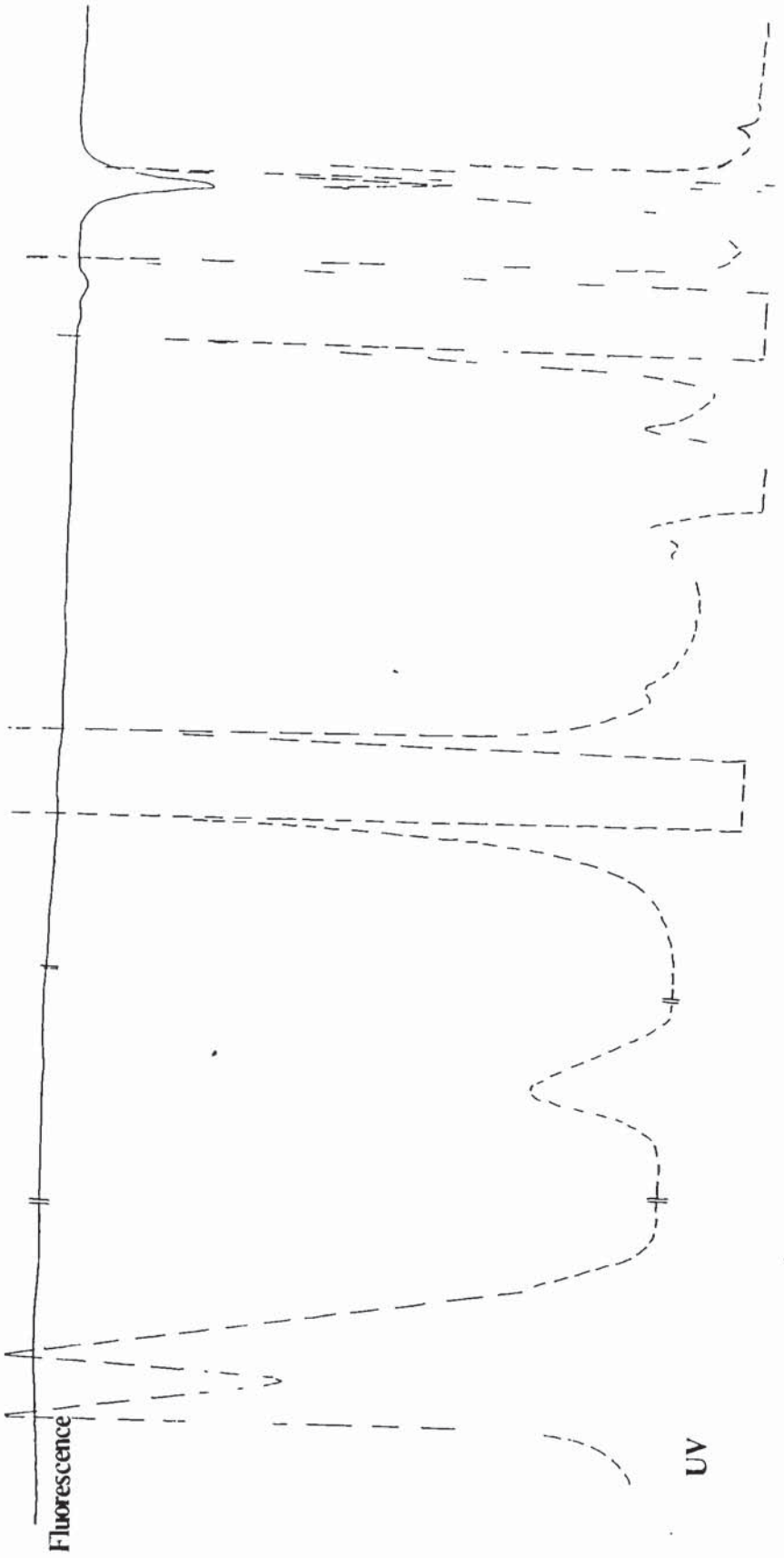


Figure 36:- Chromatogram of standard lipid mixture 1.

Figure 37:- Chromatogram of standard lipid mixture 2.

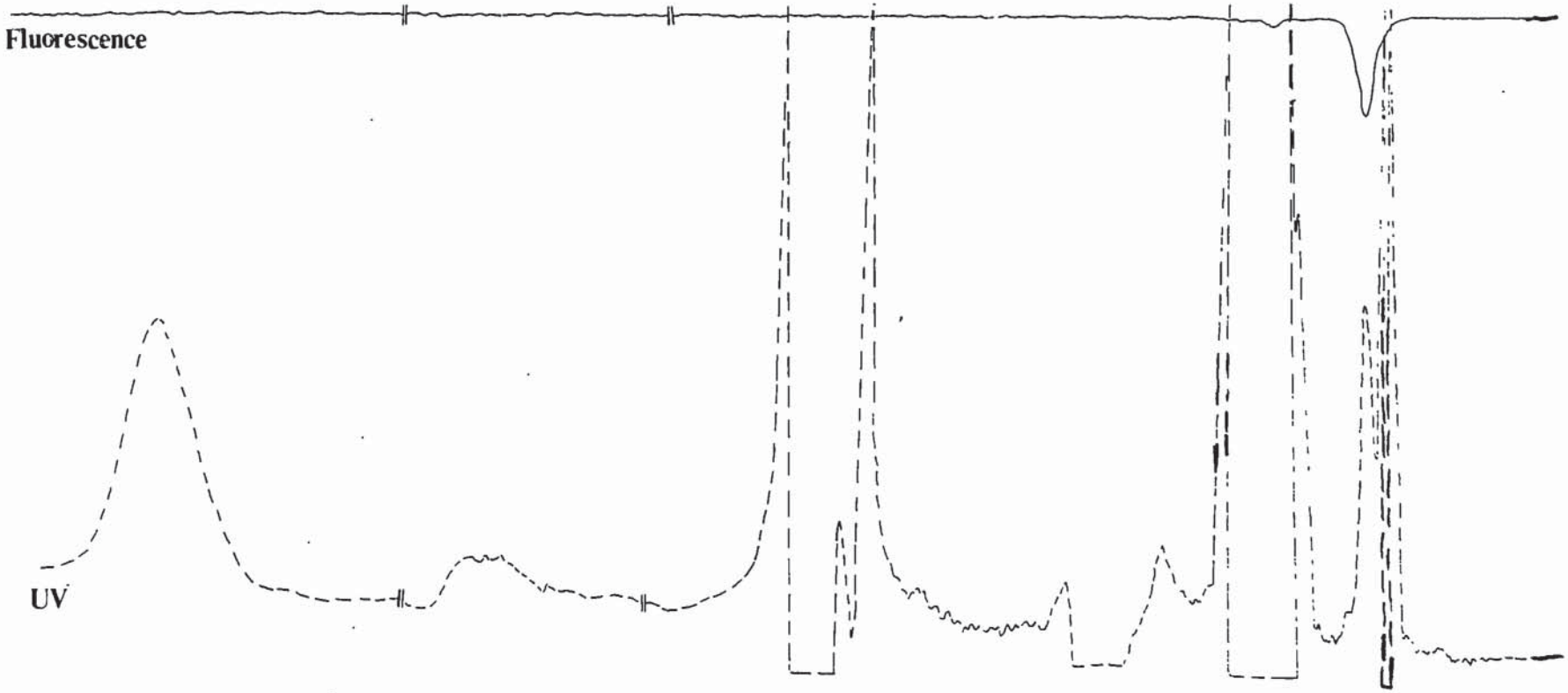
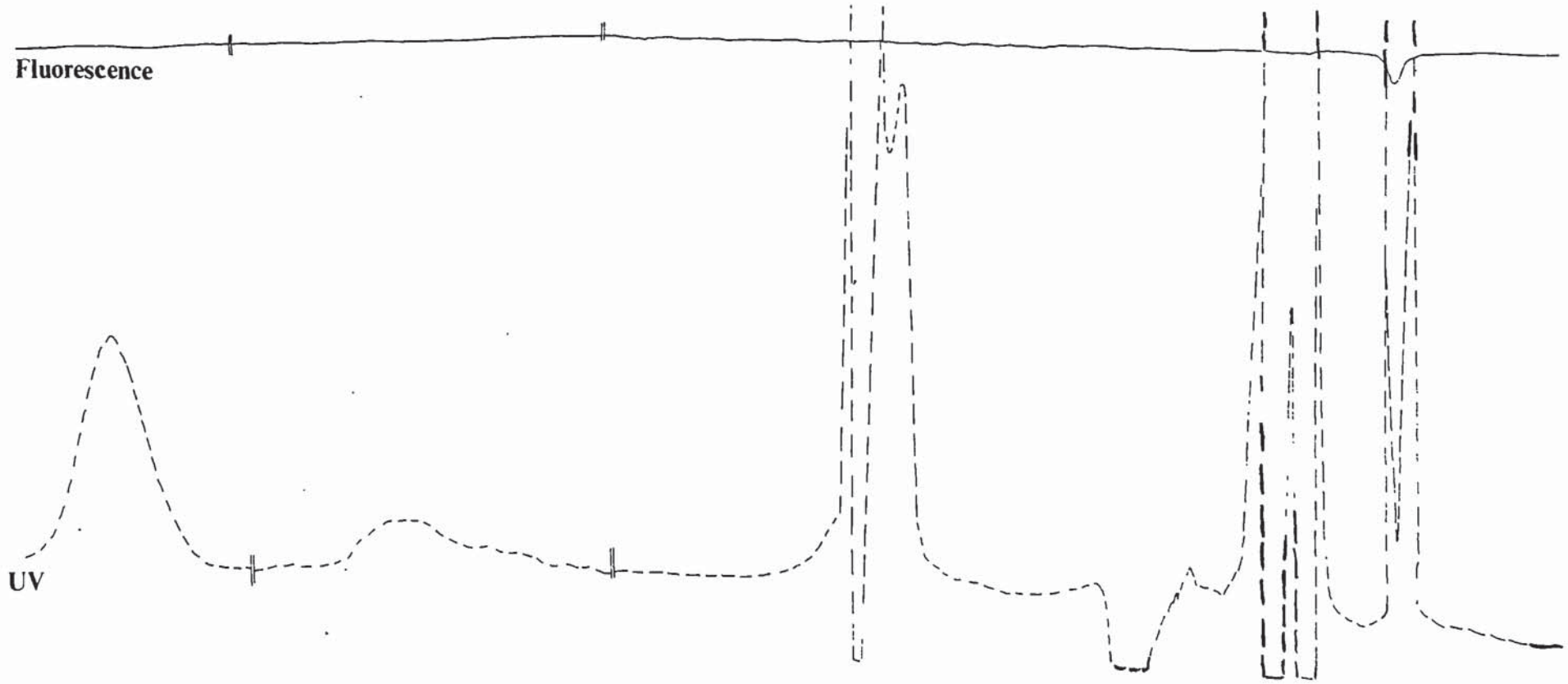


Figure 38:- Chromatogram of standard lipid mixture 3.



All the lenses analysed showed a broadly similar pattern of retention times indicating the presence of cholesterol esters, triglycerides, fatty acids, cholesterol and probably diglycerides, monoglycerides, fatty alcohols and phospholipids. There was however a variation in the quantities of these classes relative to each other. There may be an effect due to competitive adsorption, variation in the tear chemistry of the patient and due to extrinsic factors such as handling.

The chromatograms of the soft contact lenses inserted for 15 minutes have well defined patterns for both the two right eyes and the two left eyes. All four chromatograms have cholesterol esters, triglycerides, fatty acids and probably monoglycerides, diglycerides , fatty alcohols and phospholipid components. The left eye samples also have cholesterol components. The two right eye samples have comparable peak areas to each other; as do the two left eye samples. The relative pattern of peak areas between lipid classes is also the same for all four lenses i.e. triglycerides, cholesterol esters, fatty acids, cholesterol and other lipid classes. There is however a difference between the right and left eye chromatograms shown by the cholesterol component present on the left eye traces.

The hard lenses inserted for 15 minutes have retention times indicative of cholesterol esters, triglycerides, fatty acids, phospholipids and cholesterol. The peak areas of the components extracted are greater than those extracted from the contact lenses worn for 18 months by the same patient. The total lipid peak areas of the hard contact lenses are similar to those for the soft lenses, although there are more additional peaks with the former. For all the lenses inserted for 15 minutes the triglyceride and cholesterol esters are the predominant feature of the chromatogram.

The white spot deposited lenses which were analysed showed that lipoidal material can be extracted from the contact lens, but the white spot deposit remains after extraction. Both soft contact lenses showed retention times indicative of cholesterol esters, triglycerides, fatty acids and probably monoglycerides, fatty alcohols and phospholipids. The peak areas of the extracted lipoidal material varies with a decrease in the order triglyceride, cholesterol esters, fatty acids and other lipid classes. These peak areas are lower than those extracted from the tear envelope after 15 minutes.

The soft contact lens samples have more extractable lipoidal components than the excised white spot deposits, except for contact lens sample 4 which has a higher proportion of extractable lipid from the excised white spot deposit. The lipid peak areas of the lens samples are greater than those extracted from the excised white spot deposits, except for contact lens sample 4. It is also interesting to note that the extractable lipid from the lens material has a component indicative of cholesterol, whereas the excised white spot deposit extracted lipoidal material has a component which is probably a monoglyceride or fatty alcohol. There is also a variation in the amount of lipoidal material extracted from the four lens samples.

The calcium film lens extracts had a variable composition although the peak areas of the lipid components present in all three lenses were fairly similar. Lens 151 and lens 154 have cholesterol esters, fatty acid and cholesterol components, whereas lens 156 has cholesterol esters, triglycerides and fatty acids. The main lipid class is the cholesterol esters with low levels of fatty acids, triglycerides and cholesterol. The calcium film was still present after extraction.

The hard contact lenses were worn daily for 22 and 18 months and cleaned using a Boston cleaner and care solution. The chromatograms have components indicative of cholesterol esters, triglycerides, fatty acids and probably monoglycerides, diglycerides and phospholipids. There is however a difference between the right and left eye extracted lipoidal material in that the right eyes have a cholesterol component for both patients. The total lipid peak areas are similar for the right and left eyes for each patient. The contact lenses worn daily for 18 months do, however, have a lower concentration than those worn for 22 months.

It is also interesting to note that fatty acids are present in low concentrations when lipids extracted from the deposited lenses are compared to the extracted tear film. The peak areas for all these fatty acids are observed to be much lower when extracted from deposited lenses as opposed to the tear envelopes for both soft and hard lenses. In the case of the lenses where the deposit and lens were extracted separately three out of the four lenses showed similar amounts of fatty acid for both the lens and white spot deposit samples. With the calcium film lenses the amount of fatty acid was very low in comparison to the other soft lenses. This may however be due to the very high quantities of cholesterol esters observed. The low levels of the fatty acids maybe due to (a) the ease with which they can be washed off the lens during normal wear, (b) to the fact that they are so strongly adsorbed they cannot be removed without destroying the polymer matrix or, (c) because the lipids have penetrated the polymer matrix.

The composition of the lipids extracted from all the contact lenses, except the tear film envelopes, may also have been affected by the storage of the lens in saline or the

possibility of lipids being so firmly adhered to the lens that they can not be extracted. The former is likely to be a small effect due to the fact that lipids by definition are water insoluble organic molecules.

The initial results of the sterile handled lens experiments showed that lipids are deposited onto the contact lens during handling. The lipids extracted from the lens showed fewer components than those extracted directly from the skin. The concentration of the lipids from skin contamination is much less than the tear volume lipoidal mass, but this may not be true during the interfacial conversion process. The handling of the contact lens for longer periods of time did not appreciably increase the concentration of the lipoidal material deposited onto the lens. There is some variation in the composition of extracted lipids between the subjects. Thus, good care regimes capable of removal of any lipoidal contamination deposited as a result of handling as well as wear are required.

Further identification of the lipids extracted from the contact lenses and the tear film may be aided by other detectors e.g. refractive index or other techniques e.g. Gas-chromatography-mass spectrometry (GC-MS), Secondary ion mass spectrometry (Sims).

These results show the potential importance of being able to analyse the minute quantities of material deposited onto a contact lens during everyday wear and spoilation. They also highlight the variability of the lipids of individual patients ocular secretions.

CHAPTER 6.

Development of an *in vitro* model to mimic ocular spoilation.

6.1. Introduction.

In order to understand the nature of the initial events of ocular spoilation it is necessary to be able to analyse these events in conjunction with any extrinsic factors that may contribute to the interfacial conversion process. If a process is understood (e.g. blood clotting) attempts can be made to reproduce the initial stages *in vitro*. This is also true with the initial stages of ocular spoilation although the underlying mechanism is the based on speculation resulting from incomplete spoilation studies. If we can mimic the initial stages of ocular spoilation *in vitro* perhaps we will be better able to understand the process. An *in vitro* model would enable certain aspects of materials-related studies to carried out without the complications of patient to patient variation.

While animal experimentation has been used to study the effects of synthetic products on biological systems in the past, the three main animals used in contact lens research have not proven entirely satisfactory for such work. Reasons include the following :

- the rabbit has a slow blink rate in comparison with the human
- the rat has very small eyes, causing practical complications
- the monkey is both rare and expensive.

In addition to these technical problems, animal experiments are becoming less acceptable in modern society. While investigation using groups of human beings is a possible alternative, there are several problems with this type of study. These problems include the wide variation in patient tear chemistry, the need for strict control of the conditions of study and the resultant inability of this approach to date to deal with a variety

of novel polymers. An *in vitro* study however, is controlled, flexible, simple and an inexpensive solution to many of these difficulties and is becoming a fast expanding and much needed area of experimental research, in the field of biomaterials.

In designing any *in vitro* system, several criteria must be met, in order to ensure that the *in vitro* and *in vivo* situations are as similar as possible.

There are several general criteria for an in vitro model. These include the following:-

1. It must mimic the *in vivo* situation as closely as possible.
2. It must be relatively simple to operate. This minimises experimental errors, which may occur if the system is complex and thus confuse the observations.
3. It must be set up in such a way that alteration of the conditions within the model is relatively simple (for instance, changes in the concentration of solutions and in the temperature). In short, the model must be sufficiently flexible to allow comprehensive investigative work to be carried out.

In addition to these general criteria the following specific criteria need to be met for an *in vitro* model for mimicking contact lens spoilation. These are:-

1. There must be facilities for the lenses to be in contact with both the air and the tear solution.
2. There must be provision for subjecting, simultaneously, several types of lens, to the process of spoilation.
3. The *in vitro* tear solution must be comparable with tears.

4. Suitable non-destructive analytical techniques must be used in order to follow the processes that occur.

The interaction of the synthetic polymers including hydrogels with biological fluids has been widely studied in recent years. Blood contact devices and blood interaction problems have undoubtedly commanded the greatest attention, and the role of protein deposition has been recognised to be of paramount importance. It has been found that foreign surfaces immersed in plasma rapidly adsorb at least a monolayer of protein, which is partially denatured. This monolayer of protein acquired from the blood has been identified as the initial step in triggering the blood coagulation process. As a result biocompatibility studies have been based on protein adsorption. Although the aqueous tear composition in the healthy eye differs substantially from that of plasma, it does contain dissolved proteins. Thus, *in vitro* ocular spoilation studies have also been based on the adsorption of protein solutions in the form of 'artificial tears'. The majority of these studies involved using solutions of single proteins. Some other studies involved the use of a mixture of proteins [70-83]. Lipids have only rarely been used as a basis for artificial tears[84] and few studies have combined the lipid and protein [71]. Thus, in the early stages of this study the literature was very diverse in relation to the protein composition and especially the lipid composition of tears and *in vitro* models. Critical analysis of the literature and our analysis of the tear lipids involved showed none of these studies meet all the criteria for an *in vitro* model to mimic ocular spoilation. The information in this chapter relates to the development performed during the course of this study to produce an *in vitro* model which mimics ocular spoilation(but not factors associated with lid movement).

6.2. Development of an *in vitro* model system.

Tears themselves are impractical for use in an *in vitro* model, as their extraction is difficult and it is only possible to obtain a few microlitres at a time from a single person, unless tear flow is stimulated artificially. Such stimulation caused by ocular irritation, say by onions, results in a different composition of tears than is present in the eye under normal conditions. In addition to this difficulty there is a wide variation in patient tear chemistry making a constant composition of tear fluid difficult to maintain throughout the study.

The development of the *in vitro* tear fluid model used in these studies arose form work in these laboratories on the interactions between polymers and mammalian cells. Animal sera, in particular foetal calf serum (FCS) have been used for many years in tissue culture studies to supplement the cell culture media. Cell-substrate interaction studies showed that small variations in polymer structure induced for example, by treatment with sulphuric acid (which alters the hydrophilic nature of the polymer by introducing hydroxyl or sulphonate groupings into the structure) produce dramatic changes in the ability of the polymers to turn on and off cell adhesion [237-241]. Foetal calf serum (FCS) is known to optimise the conditions for BHK fibroblast growth. Under physiological conditions a serum layer is deposited during the period of cell growth. This serum layer adsorbs onto substrates such as bacteriological and tissue culture plastic regardless of the length of time the experiments run, whether from 15 minutes to 24 hours. No differences were detected, however, in the composition of the serum layer deposited on different substrates, which might have correlated with the ability of these surface to support cell adhesion and growth[231, 239]. This ability is probably related to the concentration/conformation of specific protein fragments. The rapidity of formation and composition of the lipoidal

fraction deposited during cell growth was observed to bear a marked resemblance to the deposited layer resulting from short term *in vivo* experiments involving contact lenses.

As a result of this further experiments were carried out to determine whether animal sera of this sort could be successfully used as the basis for development of a 'surrogate' tear fluid to be used in an *in vitro* ocular spoilation studies.

The following conclusions led to the belief that FCS was a suitable substitute to form the basis of a 'surrogate' tear fluid:-

1. Past research at Aston University has shown the serum layer bears a marked resemblance to components adsorbed onto synthetic surfaces,
2. It is a solution whose composition has many broad similarities to tears (see table 6.1),
3. It does not contain clotting factors, which would be an added complication if using other biological fluids,
4. It is readily available,
5. It is a naturally occurring and stable solution of a complex array of lipids. A natural system is very much more attractive for this type of study, as problems with stabilisation have already been overcome by nature itself.

Table 6.1:- Comparison of foetal calf serum (FCS) and tears.

	FCS (g/l)	TEARS (g/l)
Total protein	45-70	6.5-8
Albumin	22	3.9-3.94
Lactoferrin	-	0.81-1.48
Lysozyme	-	0.65-2.2
Total lipids	1.6-2.3	1.96-2.4
Cholesterol	0.5	0.08-0.32
Ash	7.2	10.5
Water	95.22%	98.2%
Total solids	48	18

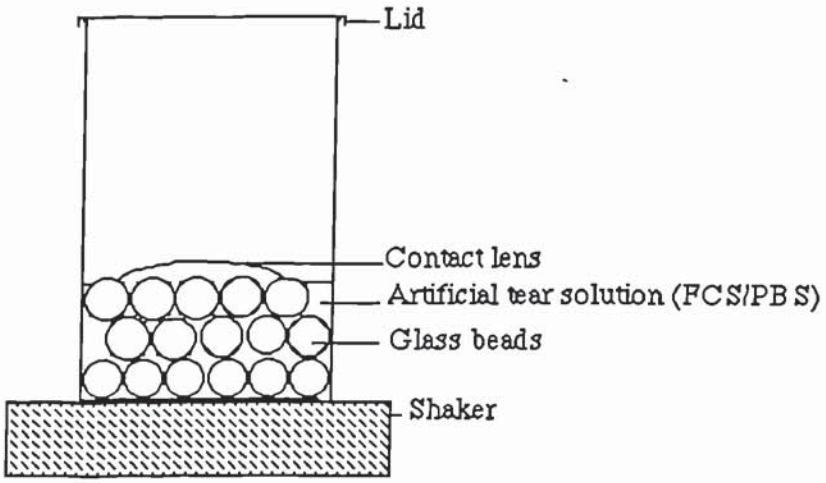
(Figures obtained from references 29, 85 and 242, the values for FCS being an average of those given for commercially produced and specially produced sera. Widely different values are quoted in the literature for the various components in tears due to patient tear chemistry variation, hence the total protein, lipid and ash components are more reliable indicators.)

In order to bring the total solids to a comparable level the foetal calf serum used in this model was diluted 1:2 (v/v), with phosphate buffered saline, (PBS) of pH 7.4. This dilution was found to work well and allowed scope for 'spiking' the model solution with individual components such as lysozyme, lactoferrin, fatty acids, mucins etc. The point of this was to produce a solution more comparable with that of tears and additionally it

allowed the effect of artificial enhancement of individual components to be investigated.

The experimental arrangement was then set up as shown in figure 6.1. Glass beads were placed in the vials, in order to provide an uneven surface on which the lenses would sit. This was to allow contact with both air and the tear solution, the latter being pipetted in to a level just below the "upper surface" of the glass beads. The prepared vials were then placed on a shaker. This was to enhance the air and tear contact with the lenses.

Figure 6.1:- Arrangement for the shaker model.



The main observation during the time that this initial model was running was that within a few hours of introducing the FCS into the vials, it had turned cloudy, suggesting that the protein components were denaturing. Further evidence that denaturation was taking place was the noticeable smell of hydrogen sulphide (H_2S) on removal of the vial lids. This suggests that the disulphide linkages of the protein were being broken down. This problem would not occur in the eye, where the biological fluids are naturally stable and constantly being replenished. Thus, in order to ensure that the model mimics the eye

as well as possible, this denaturation had to be prevented. The following suggestions were made as to why the FCS had turned cloudy:-

1. The pH of the saline may have altered, due to an inefficient buffer
2. The FCS may not be stable at room temperature
3. The shaking motion may have disrupted the protein structure, leading to denaturation
4. The free hydroxyl groups on the surface of the glass may have been attracted the proteins, which, once attached, would have become denatured, then shaken back off into the solution.

These aspects of the model were thoroughly examined, in an attempt to prevent denaturation and the following observations were made:-

1. The pH of the FCS in the vials had remained at 7.4.
2. After leaving a sample of FCS at room temperature for two days, it had not turned cloudy, showing that it is a stable solution at ambient temperature.
3. The gentle shaking motion at which the model had been left did not denature a sample of FCS placed on the shaker for two days, although it did turn cloudy with very much more vigorous shaking.

Thus, it was deduced that the glassware was the causative factor in this destabilisation process, by adsorption of the protein onto the surface, followed by denaturation.

A second model was devised, exactly as the first, except that, before use, the glassware was soaked for twenty-four hours in a phosphate-free soap and thoroughly

rinsed. It was then left to stand for approximately ten minutes in Repelcote, a water repellent which produces a silicone coating on the glass, rendering it inert to protein adhesion. It was finally rinsed thoroughly in distilled water. The apparatus was set up once more. Within only a few hours of setting up the model, the serum had again turned cloudy.

A further suggestion put forward to explain the denaturation of the protein was that the natural antibacterial agents in the tears, namely, lysozyme and lactoferrin, are completely absent in FCS, (table 6.1). Consequently the denaturation may have been due to microbacterial contamination in the vials. This being the case, the control of bacteria in the *in vitro* system must be achieved by some other means.

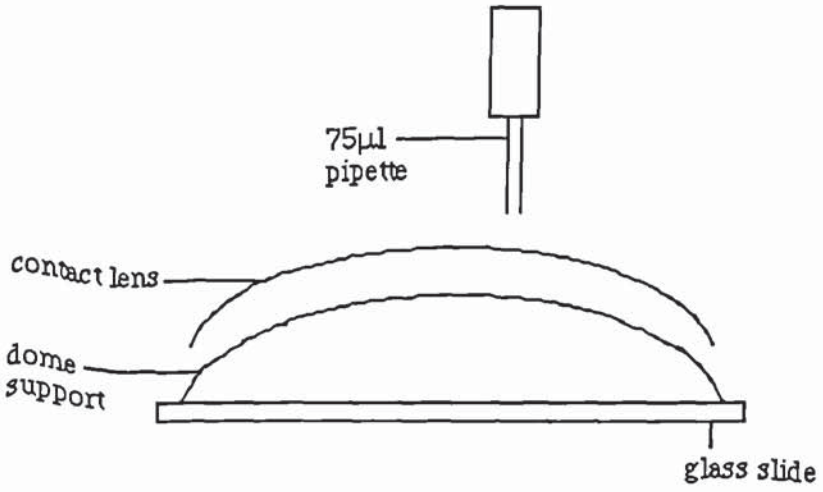
A third model was set up, this time ensuring that all the glassware and contact lenses were disinfected thoroughly in two percent hydrogen peroxide solution and then rinsed, prior to use. For the contact lenses, this was achieved by the use of the Septicon System:- the lenses were placed on the palm of the hand and two drops of Lensept added. It was then rubbed between the palm and the finger and positioned in the lens container, filled to the mark with Lensept. It was left for approximately thirty minutes and then transferred to a second container, containing a platinum disc. This time the container was filled to the mark with Lensrins. The lens was left in this solution for at least six hours, in order to remove all traces of the hydrogen peroxide.

In addition to this procedure, the lenses were subjected to the Septicon treatment after surface analysis, before being returned to the vials. No cloudiness was observed in

the vials, once this sterilisation procedure had been effected.

In addition to this 'shaker' model a second form of the model was developed. This is the 'drop and dry' model which was intended to act as a complement to the previously described *in vitro* system. Its purpose was to show if exaggeration of the evaporation of the tear film, would cause deposition to be enhanced, or to occur at a greater rate. Thus a system was produced which exaggerates this drying process. The experimental arrangement is shown in figure 6.2. Lenses of the same four types, as before, were placed on the dome supports and 75µl of diluted FCS were pipetted directly onto the lens surface. This was then left at ambient temperature for 24 hours to evaporate and then a second "drop" of FCS was added. This process was repeated every day.

Figure 6.2:- Experimental arrangement of the 'drop and dry' model.



6.3. Monitoring techniques.

Once the *in vitro* system was developed successfully, it was used in conjunction with analytical techniques developed to analyse *in vivo* spoilt lenses. These techniques are discussed in chapters 2, 3 and 4 and include contact angle measurement, electrophoresis, high performance liquid chromatography and fluorescence spectroscopy. Extraction techniques for the independent removal of the adsorbed protein and lipid were used. The protein extracts from the lenses in the *in vitro* model were to be subjected to electrophoresis and the lipid extracts to HPLC. Several standards and a sample of FCS were also to be tested to identify the FCS components which had adsorbed onto the lenses and to analyse their relative concentrations.

Many contradictory theories exist concerning the mechanism of spoilation[8,53,84,148]. The *in vitro* system was used to study the initial events in the biological interfacial conversion process. This involved increasing the concentration of a major tear component in each of several different vials, followed by observation of the resulting rates of spoilation, may then lead to the identification of the species vital to the initial stages of the deposition process. The degree and relative rate of deposition could be followed, as before, using fluorescence spectroscopy and contact angle measurement.

Four types of lens were analysed Kelvin Eurothin (polymacon), Cooperthin (polymacon), Permalens (perfilcon-A) and Permaflex 74 (sufilcon-A). The compositions of these lenses are given in chapter 2. The previously developed extraction procedure using 3ml of methanol on a shaker for 30 minutes for lipid extraction and sodium dodecyl sulphate (SDS), a surfactant, was used to extract protein from the lens surfaces. Here the

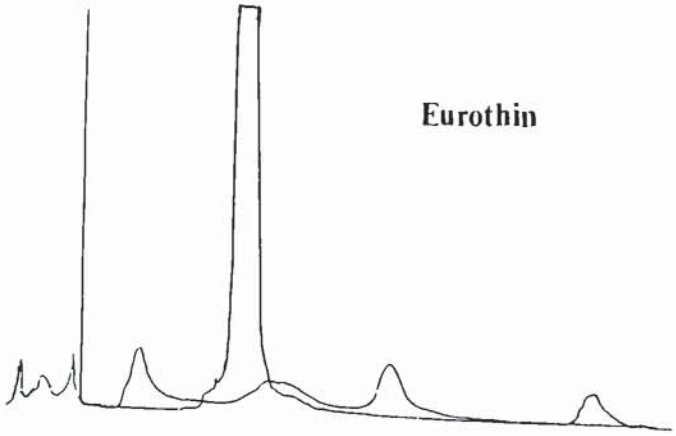
lenses were placed in a 1% solution for sixty minutes on a shaker, with slight warming.

6.4. Use of the model with commercial lenses : Results.

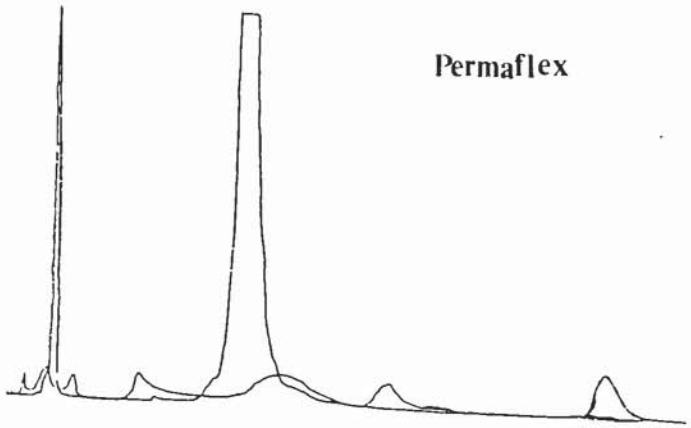
The lipid extracts were run on the high performance liquid chromatography (HPLC), along with an extract of FCS , in order to show whether the peaks obtained from the lens extracts were due to components of the *in vitro* tear solution. *In vivo* spoiled lenses were also subjected to this procedure, in order to compare the *in vivo* and *in vitro* situations. The protein extracts and a sample of FCS were placed on a Serum Protein (SPE) gel and subjected to electrophoresis.

Fluorescence spectra were run at 300nm and 360nm. Both forms of the *in vitro* model showed that fluorescence intensity increases with exposure to FCS . Initial spectra and those recorded after three weeks in the drop and dry model are shown in figure 6.3 and figure 6.4. Fluorescence emission was recorded from 200 to 800nm in all cases, the first major peak on the spectra being the chart mark at 200nm. The emission intensity is directly proportional to the area under the peaks. The emission around 350 - 400nm suggests that the Permaflex lens has adsorbed more fluorescent material onto its surface than the other lenses. The Permalens exhibits the second most obvious fluorescence, while the other two lenses show very little change. The overall changes in the lenses in the shaker model, were the same as indicated in figure 6.5, which shows the Permaflex model three lens, after two weeks exposure to the system. The other three types of lens change very little.

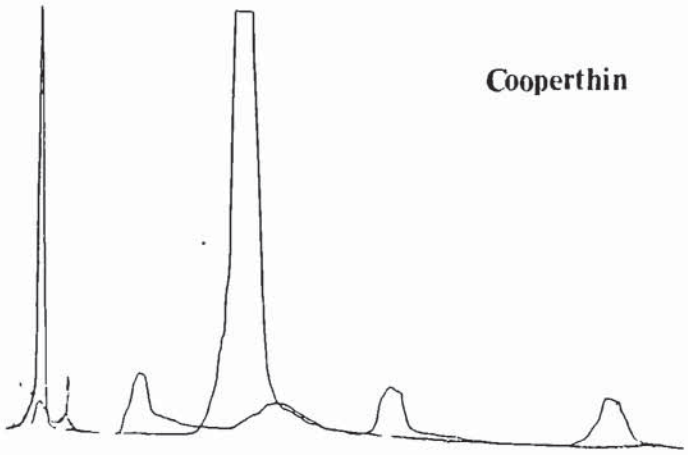
Figure 6.3:- Fluorescence spectra of the lenses before subjection to the *in vitro* model.



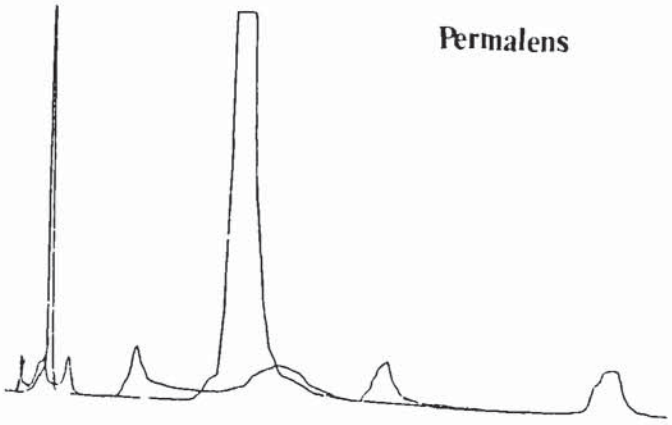
Eurothin



Permaflex



Cooperthin



Permalens

Figure 6.4:– Fluorescence spectra of the drop and dry lenses after three weeks exposure to foetal calf serum (FCS).

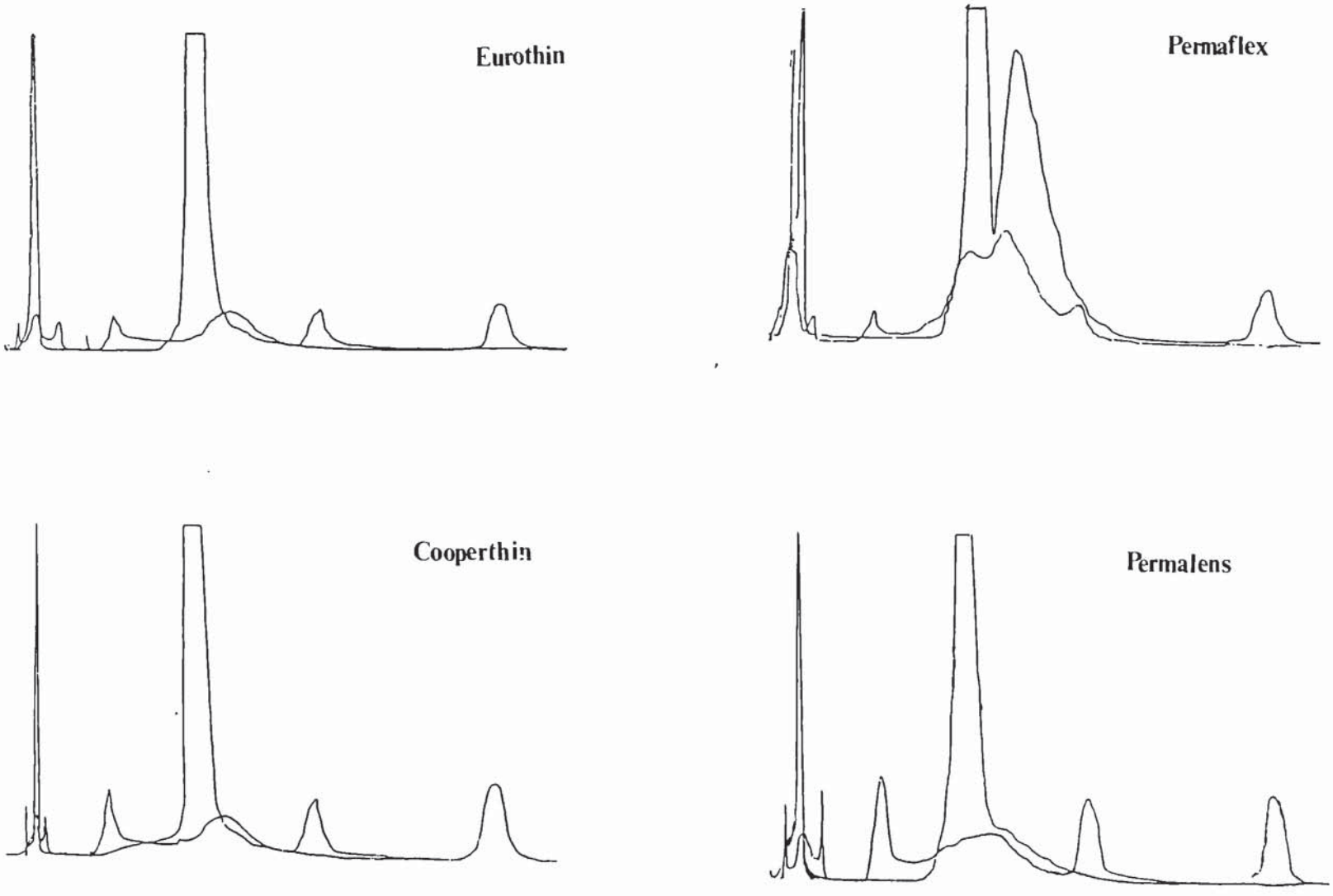
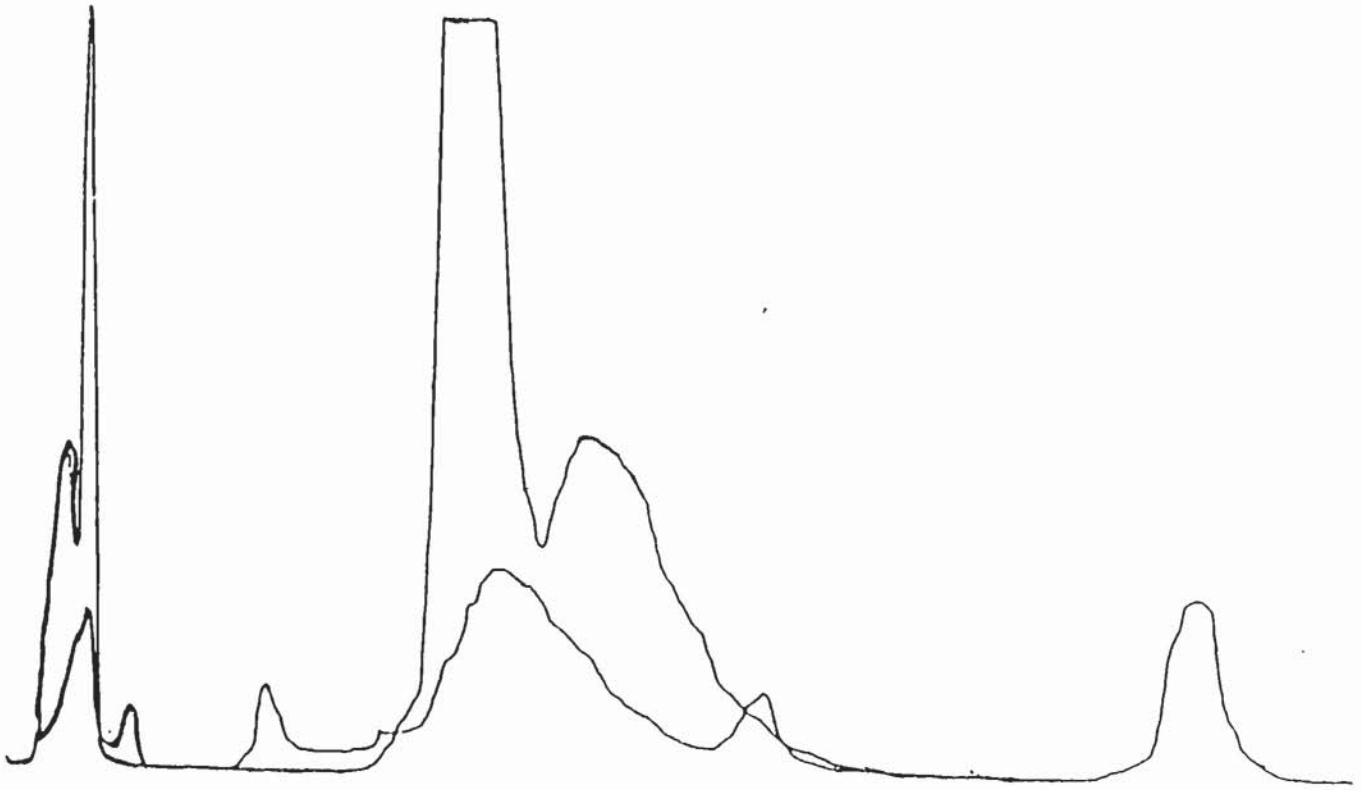


Figure 6·5:- Fluorescence spectra of the model three Permaflex lens after two weeks exposure to foetal calf serum (FCS).



Comparison of these spectra with that obtained for FCS, (figure 6.6), indicates that there are components in this solution which fluoresce at the same wavelength as the species present on the contact lens surfaces, (350 - 400nm). This is evidence that the fluorescence on the lenses originates from the FCS.

Although fluorescence spectroscopy was not used as an absolute analytical technique in this study, the spectra were analysed further, by comparison with the spectra for albumin and globulins (figure 6.7), two major proteins in FCS. The similarity of these spectra indicates the possible origin of the fluorescence obtained on the *in vitro* spoiled contact lenses.

Figure 6·6 :- Fluorescence spectrum of foetal calf serum.

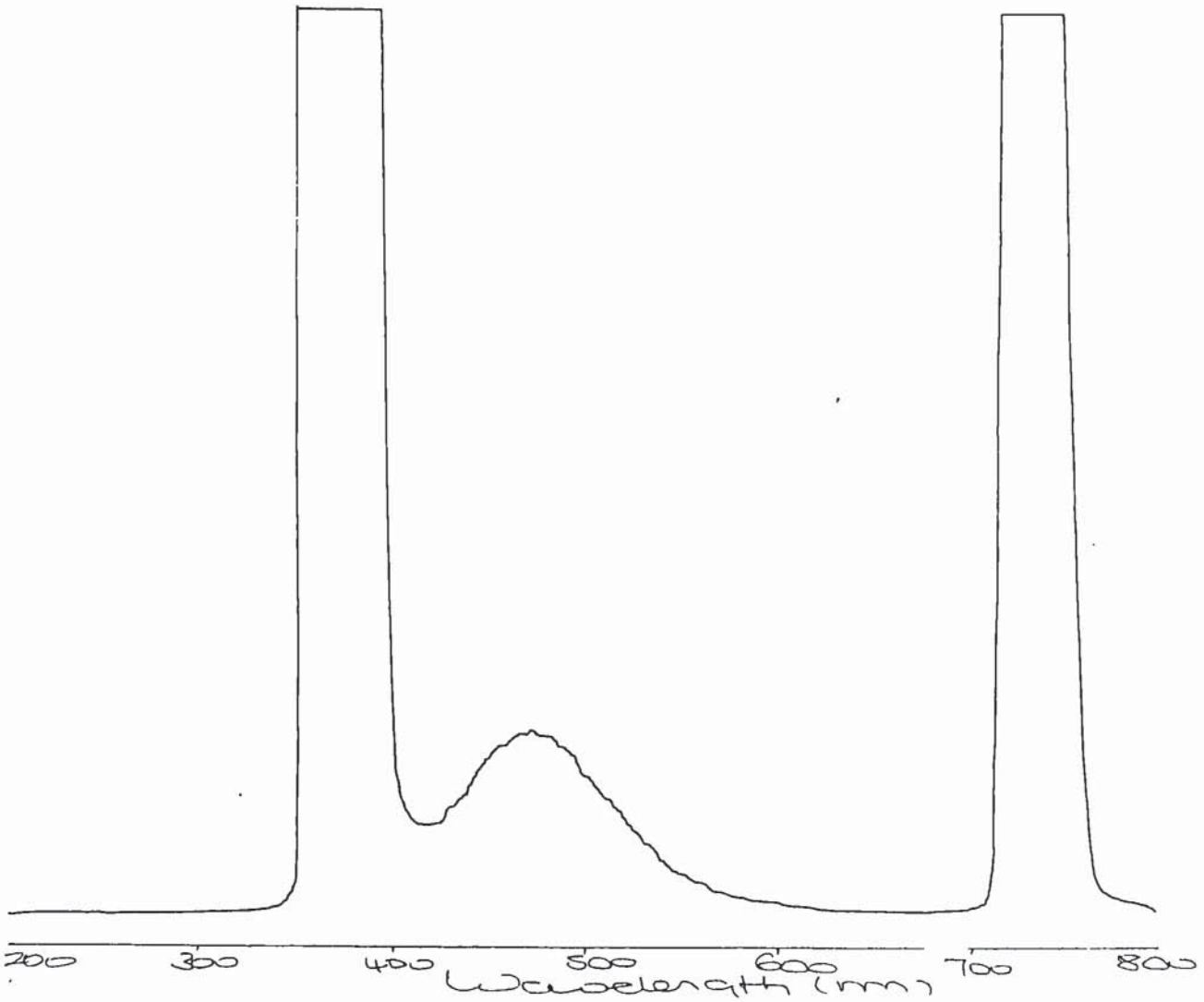
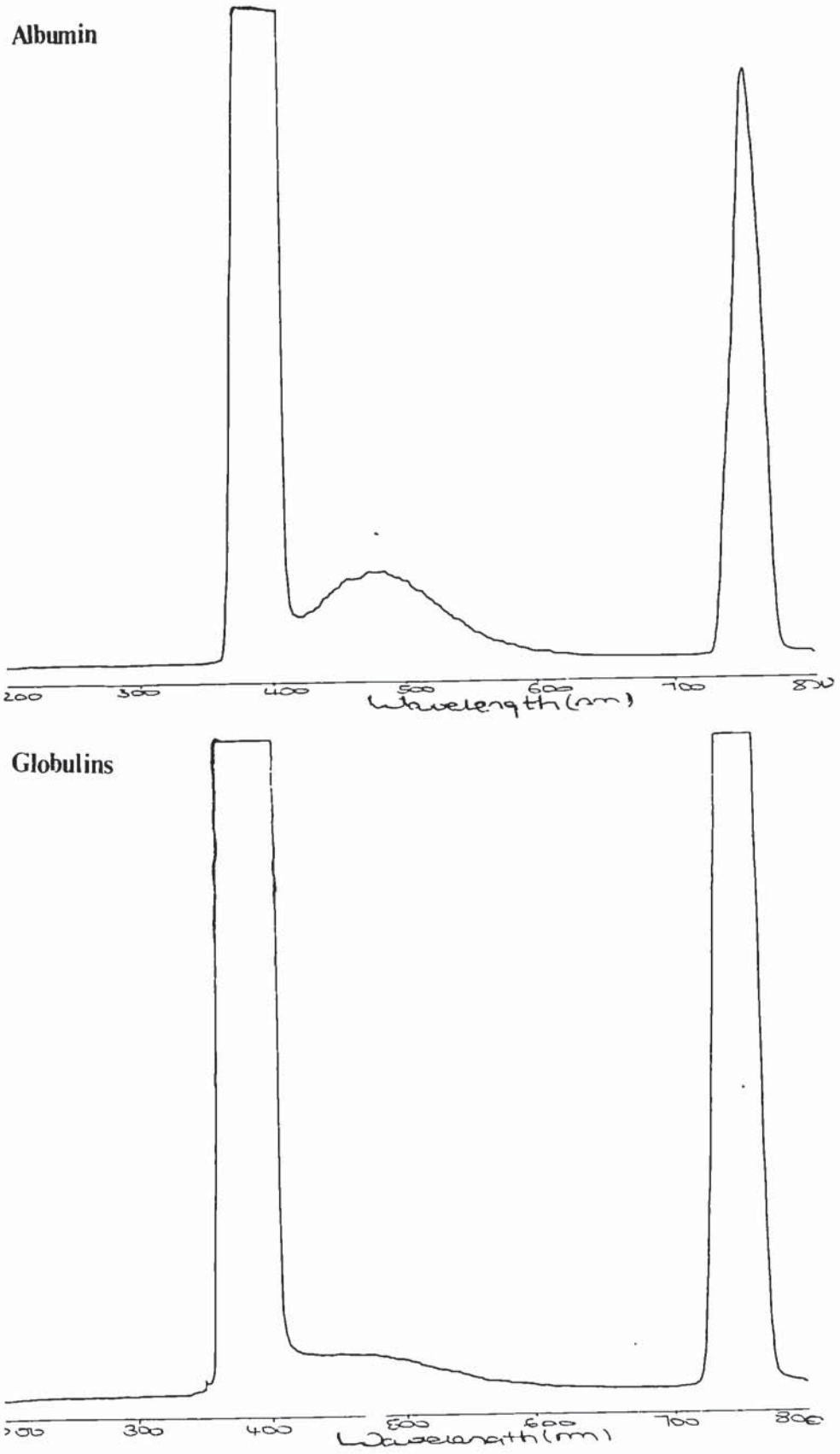


Figure 6·7:- Fluorescence spectra of albumin and human-γ-globulins.

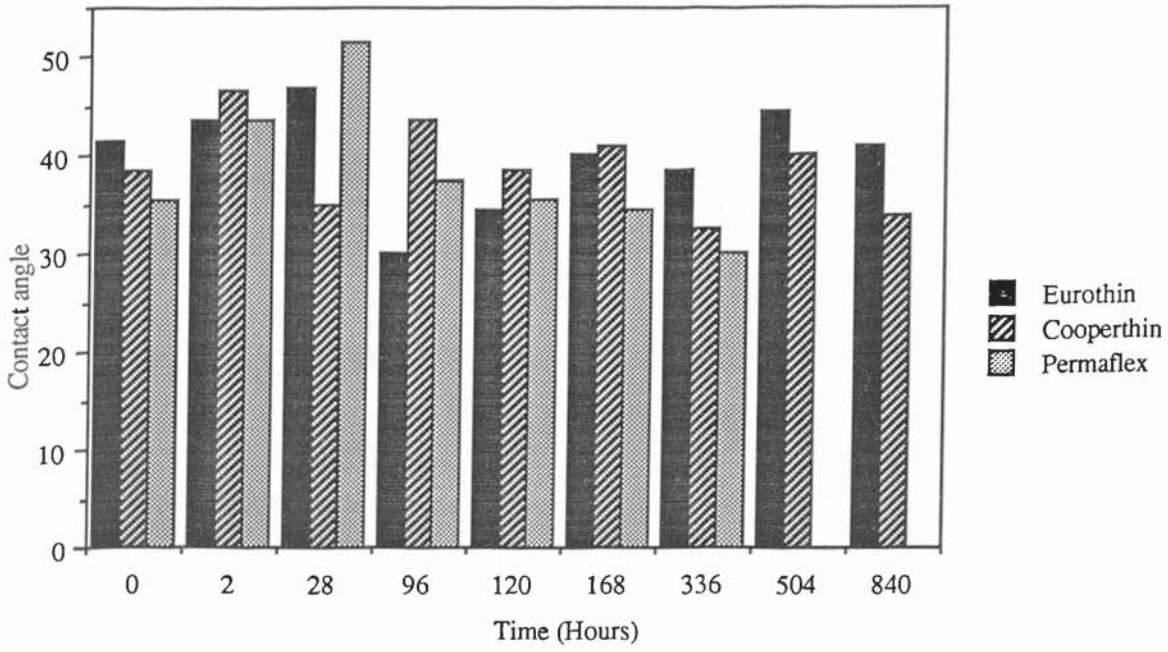


The contact angle measurements for the model three lenses are presented in table 6.2. These results do not appear, at first glance, to show any distinct trends. A closer examination of the values for the Permaflex (surfilcon-A) lens, however, shows that the contact angle, which initially, increases slightly, subsequently decreases overall, the final value, after three weeks in the model, being 0^{o} for this lens. These changes are shown in figure 6.8

Table 6.2:- Contact angles, in degrees, for the anterior surfaces of the shaker model lenses. (Equilibrium advancing angles ± 2).

Time in model	Eurothin	Cooperthin	Permaflex	Permalens
0 HOURS:	41.5	38.5	35.5	49.0
2 HOURS:	43.5	46.5	43.5	48.0
28 HOURS:	47.0	35.0	51.5	-
4 DAYS:	30.0	43.5	37.5	-
5 DAYS:	34.5	38.5	35.5	-
1 WEEK:	40.0	41.0	34.5	-
2 WEEKS:	38.5	32.5	30.0	-
3 WEEKS:	44.5	40.0	0.0	-
5 WEEKS:	41.0	34.0	0.0	-

Figure 6.8:- Contact angles for the anterior surfaces of the shaker model.



This suggests that, as spoilation takes place, the lens surface becomes more hydrophilic in nature. In the past, it has been thought that the wettability would decrease as non-polar components build up on a synthetic surface, and thus that the hydrophilic character of the surface would also decrease. However, more detailed recent study has shown that as the deposition occurs in discrete areas, it is not the whole surface which becomes more hydrophobic. In this case, a sessile drop, which is a large volume of liquid on the surface, will bridge the areas of hydrophobicity and not show the same behaviour as the relatively thin tear film, which may break up over these patches of hydrophobic character [243]. The mechanical spreading pressure of the drop, causes it to spread out to cover as much of the hydrophilic area as possible and hence spoilation does not directly lead to an increase in contact angle.

After extraction of the lipid components from the *in vitro* spoiled lenses, the fluorescence spectra of the lenses were recorded. These show that the intensity of fluorescence decreases after the extraction, thus suggesting that some species had been removed from the surface, and, if this was lipid, that some of the lipid component is fluorescent. The intensity of fluorescence was found to decrease further after extraction of the protein by SDS. The fluorescence spectra of the shaker model Permaflex (surfilcon-A) lens are given, after, first, the lipid extraction, and second, the protein extraction (figure 6.9).

It can be seen from these results, however, that even though much of the fluorescence has been removed by these extractions, there is still some fluorescent species remaining on the lens, which must be much more tightly bound to the surface than the other components.

The results of the lipid analysis shows the chromatographs from an *in vivo* spoiled Permaflex (surfilcon-A) lens, a shaker model Permaflex (surfilcon-A) lens and a drop and dry model lens (figure 6.10). These chromatograms were compared to the FCS and lipid standards run during the HPLC analysis of *in vivo* lenses (see table 4.2, chapter 4).

Figure 6.9: Fluorescence spectra of of a shaker model Permaflex lens after lipid extraction and (ii) protein extraction.

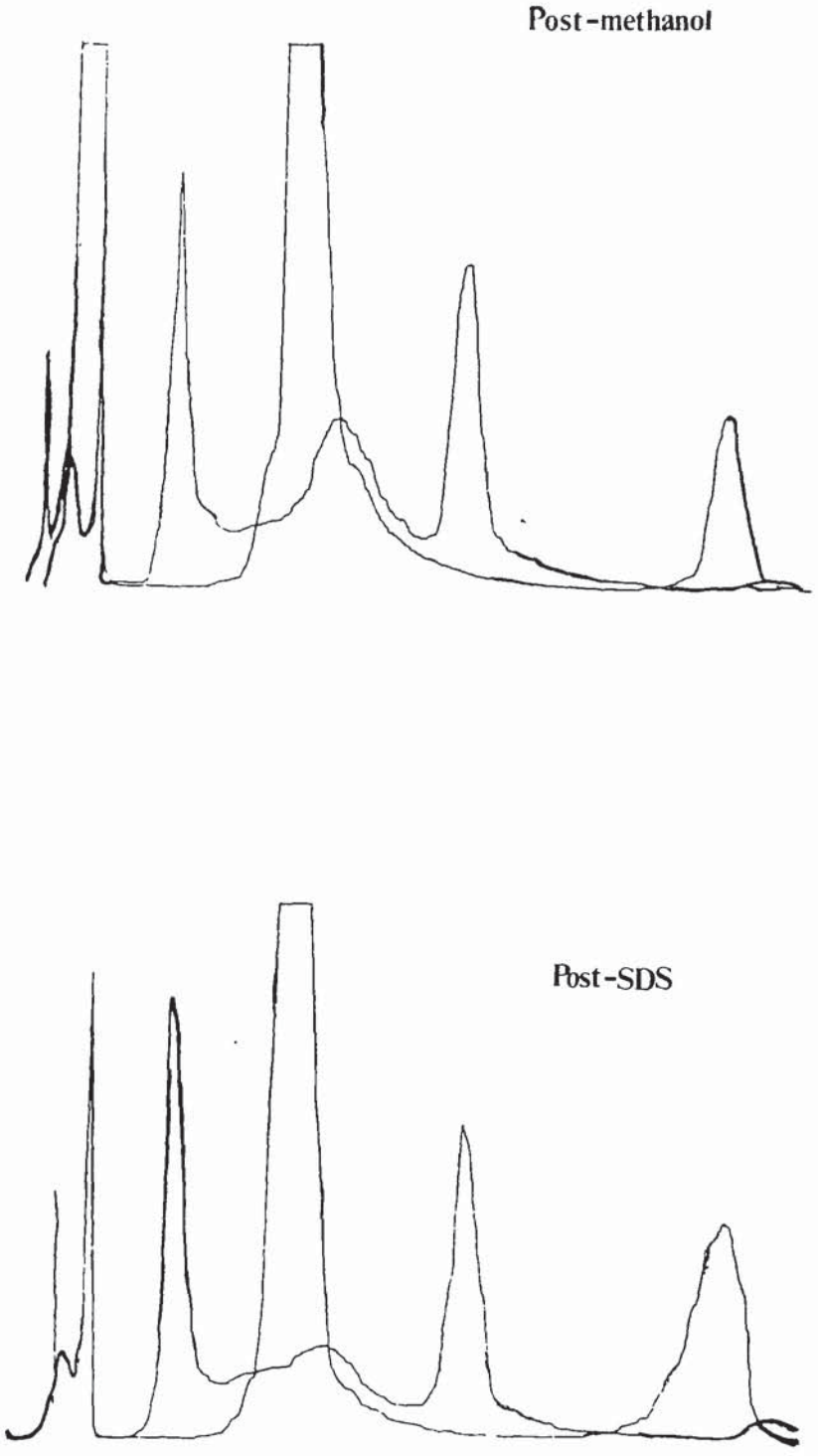
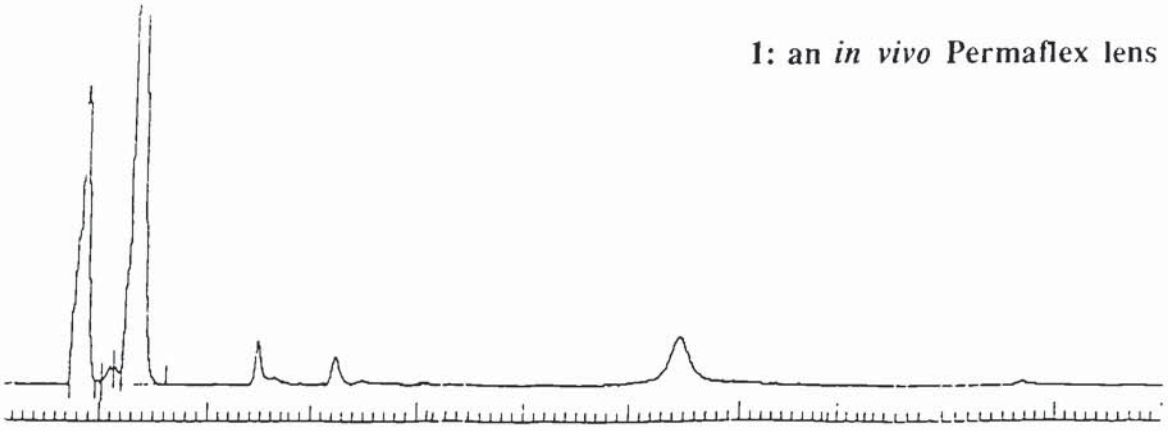
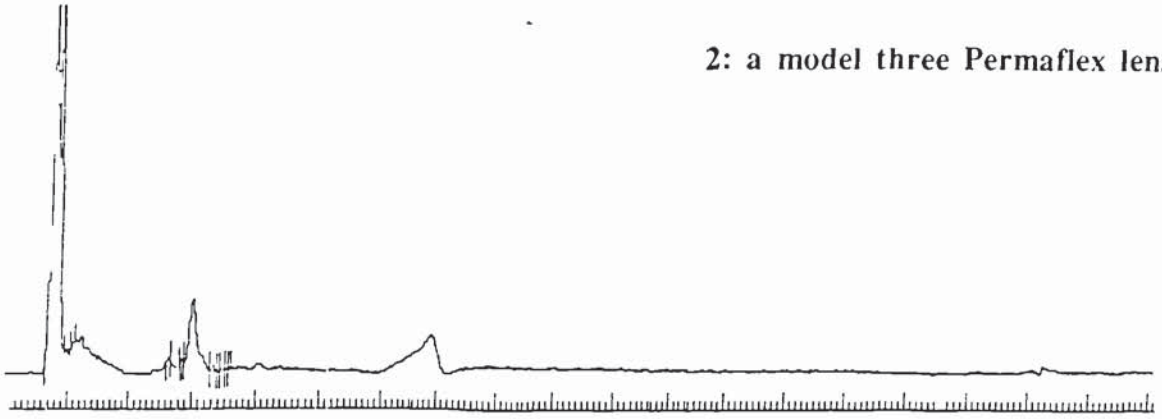
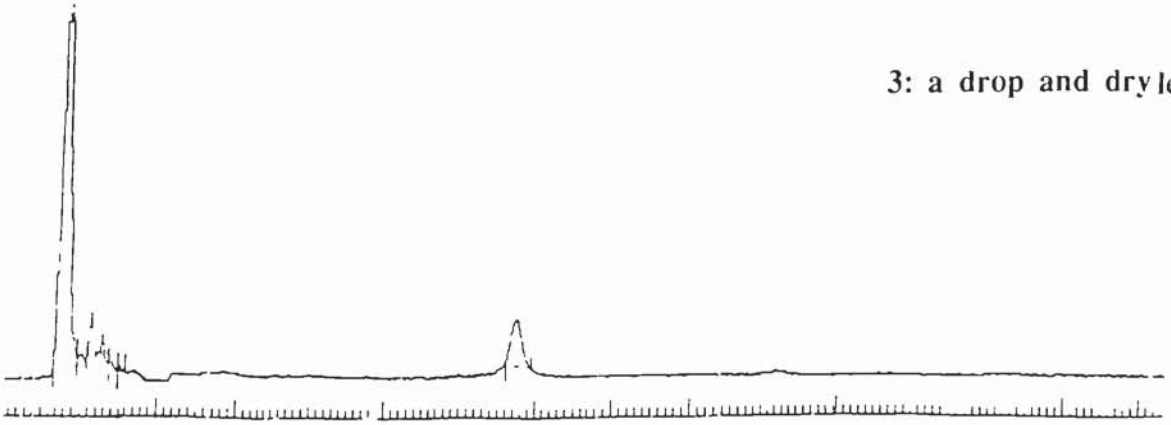


Figure 6.10: Chromatograms of the lipid extracts from 1: an *in vivo* Permaflex lens 2: a model three Permaflex lens and 3: a drop and dry lens.







As a result of this comparison the following observations were made:-

1. The extract from the *in vivo* spoiled lens is very similar to those of the *in vitro* spoiled lenses. This shows that similar lipids have been adsorbed in the two cases and that FCS therefore seems to be an ideal solution for *in vitro* study, because of its apparent similarity to tears.

2. The lenses appear to have all the above components adsorbed onto their surface to some degree, except for cholesterol. There is a peak at approximately 650 seconds. This is thought to be a monoglyceride.

3. The shaker model lens appears to have an increased concentration of adsorbed fatty acids, compared with that in FCS itself. This is absent in the drop and dry lenses.

The separation of the proteins in the SDS extracts of *in vitro* spoiled lenses, standards, and FCS, showed that FCS does not contain lysozyme and that the silver staining technique appears to be sufficiently sensitive to detect the small amounts of protein in these samples. It was also be seen that in the case of the *in vitro* lens extracts, the protein concentrations are too low to be detected by this technique. Fluorescence spectroscopy confirmed, however, that the extractions had been successful, by displaying the appropriate emission peaks.

Once these initial studies proved successful the FCS was modified to study the essential components of ocular spoilation. Cholesterol and its esters constitute a relatively large fraction of the lipid component of tears, as do the fatty acids. These two components are each thought to play a major role in the process of spoilation, as a result of work at Aston University and at other institutions [143,148]. Thus, it was decided to test the effect of

at the highest concentration in tears, and so, as there is no lysozyme in foetal calf serum, it was suggested that lysozyme should be added to all the solutions, in order to bring the *in vitro* system closer to the situation in the eye.

To determine the extent to which the concentration of these components should be raised, the composition of foetal calf serum was compared with that of the extracts, obtained from past research on *in vivo* spoiled hard contact lenses - in the case of the lipids. This composition was used, in favour of that of tears, because it has been suggested that selective adsorption of the tear components in question occurs [148]. If this is the case, the relative concentration of the lipid component should be higher in these extracts than in tears. Thus, these concentration values may provide a reasonable estimation of the extent to which concentrations can be modified in FCS, without going too far and altering the nature of the processes occurring here.

The fatty acid chosen for this initial investigation was linolenic acid, while cholesterol palmitate was chosen to represent the cholesterol and cholesterol esters component. Linolenic acid has a structure with a reasonable amount of unsaturation and so was intended to help verify the hypothesis that the unsaturated fatty acids polymerise to the surface of the contact lens and in doing so initiate interfacial conversion.

Previous HPLC analysis of extracts from hard contact lenses, carried out at Aston University found the fatty acid concentration to be approximately 20% of the total lipid component.

Mass of lipid in foetal calf serum = 0.231g/100ml

Thus, to make the compositions equivalent, the concentration of fatty acid should be 20% of 0.231g/100ml, which is 0.0462g/100ml.

There is already approximately 0.005g/100ml of fatty acid present in foetal calf serum

Thus, to 100ml of FCS, 0.05g of linolenic acid must be added.

Cholesterol and its esters form approximately 40% of the total lipid portion of the extracts from hard contact lenses.

Mass of lipid in foetal calf serum = 0.231g/100ml.

Thus, to make the concentrations equivalent, the concentration of cholesterol should be 40% of 0.231g,which is 0.0924g/100ml.

There is already 0.045g/100ml of cholesterol and esters present in foetal calf serum.

Thus, to 100ml of FCS, 0.05g of cholesterol must be added.

The lysozyme concentration was increased in all the solutions, to ensure that the foetal calf serum was more comparable with tears.

With reference to table 6.1:-

Mass of total protein in tears = 0.7345g/100ml

Mass of lysozyme in tears = 0.1000g/100ml

Therefore, lysozyme constitutes 13.6% of the total protein of tears.

The total mass of protein in foetal calf serum is 3.91g/100ml, so in order to make these solutions equivalent, the concentration in FCS should be 13.6% of 3.91g/100ml,which is 0.532g/100ml

Thus, to 100ml of FCS, 0.5g of lysozyme must be added.

Several samples of FCS were, thus, modified by various combinations of the above components to demonstrate whether or not any of them played an important role in the spoilation process, either individually or in combination.

Solutions were to be prepared, by first dissolving the components to be added in 0.5ml of FCS and then adding this to the bulk of the solution. Finally, the 10ml were to be diluted with 20ml of phosphate buffered saline. The composition of each solution is shown in table 6.3.

Table 6.3:- Compositions of the modified FCS solutions.

Solution	FCS(ml)	PBS(ml)	Lysozyme(g)	Linolenic Acid (g)	Cholesterol Palmitate (g)
1.	10	20	0.05	-	0.005
2.	10	20	0.05	0.005	-
3.	10	20	0.05	0.005	0.005
4.	10	20	0.05	-	-
5.	10	20	-	-	-

These solutions were to be used in the final shaker model, being placed in sealed, sterilised vials with the lenses and glass beads and left on a shaker. However, several difficulties were encountered during the preparation of the solutions, the first of these being that cholesterol palmitate is insoluble in aqueous media. It was suggested that it may be possible to dissolve it in the linolenic acid, before addition to the 0.5ml of FCS. However,

it appeared that the smallest volume of linolenic acid which would dissolve the cholesterol palmitate was six times the mass which had been calculated for these solutions, (i.e.0.03g). Thus, a second cholesterol ester was examined for its aqueous solubility. Cholesterol linoleate was also found to show similar properties to the palmitate. It seemed then that the only possible way to include the cholesterol ester in the solution, was to dissolve it in 0.03g of the fatty acid, before addition to the bulk of the solution, despite this greatly increased fatty acid concentration. Therefore, several solutions were prepared, one with cholesterol palmitate dissolved in the linolenic acid, and several others with various concentrations of the acid. This was an attempt to see if there is a limit to the amount of fatty acid which can be added to FCS without destabilisation. In addition, one solution was prepared without the phosphate buffered saline (PBS), to demonstrate whether FCS can be modified on its own (see table 6.4).

Table 6.4:- Composition of the second modification of foetal calf serum.

Solution	FCS(ml)	PBS(ml)	Lysozyme (g)	Linolenic Acid (g)	Cholesterol Palmitate (g)
6.	10	20	0.05	0.03	0.005
7.	10	20	0.05	0.03	-
8.	10	20	0.05	0.015	-
9.	10	20	-	0.005	-
10.	10	-	0.05	0.005	-

The second problem was uncovered when, after less than one day on the shaker,

solutions 1, 2 and 3 were found to contain a white precipitate. After two days, solution 4 exhibited the same conditions. As solution 5 had remained clear, this suggested that bacterial contamination was not likely to be the problem here. The most probable suggestion for this precipitation was that somehow the added components were destabilising the otherwise naturally stable solution. However, in order that contamination could be entirely ruled out here, the phosphate buffered saline was boiled to destroy any bacteria which may be present and all the glassware was thoroughly re-sterilised. This did not however resolve the problem.

A further series of 'spiking' modifications of FCS were tested. The added lipid and proteins were dissolved in the required volume of PBS. The FCS was then added to this solution and agitated gently to thoroughly mix the two solutions. These tests resulted in successful spiking of the FCS:PBS 1:2 (v/v) solution with lysozyme, cholesterol, cholesterol oleate, albumin, linoleic acid, cholesterol palmitate; singly or in combination. No precipitate was observed after 24 hours at room temperature on a shaker. The surrogate tear fluid was replaced in the shaker model every 24 hours to ensure a constant supply of undenatured protein and lipid components.

6.5. Discussion of results.

Thus an *in vitro* model was developed which meets the criteria laid down at the start of this chapter, including the interfacial parameters of an air gap, tear film thinning on the lens surface, a constant tear fluid composition etc..

Thus, it can be seen that this *in vitro* model mimics the process of spoilation well.

The lenses which were subjected to the system for several weeks, showed that components of the FCS had adsorbed onto their surfaces.

The fluorescence spectra of the model lenses demonstrate that Permaflex (surfilcon-A) lenses spoil to a far greater extent than the other three types of lens. The Permalens (perfilcon-A) showed the next most marked effect, while Eurothin (polymacon) and Cooperthin (polymacon) spoiled very little.

Fluorescence spectroscopy was found to be a useful technique for this type of study, as it provides a non-destructive method of monitoring the spoilation process in the *in vitro* system.

Lysozyme is not vital for the process of spoilation, as has been suggested in the past, as lipids and proteins are adsorbed onto the surface of the lenses, despite its absence in foetal calf serum (FCS).

The contact angle measurements suggest that the lens surface becomes more hydrophilic with spoilation. Although deposition occurs in localised areas on the lens surface, causing small patches of hydrophobicity, a sessile drop can bridge these patches and so tends to spread out across the surface of the lens. This produces a decreased contact angle which indicates that the surface is more wettable.

This simple model can therefore be used to investigate the role of lipids in the biological interfacial conversion process. The effect of lipid and protein components on the

initial physico-chemical events; the effect of daily or extended wear regimes; the effect of material composition including novel polymers; the effect of extrinsic factors including surfactants, enzyme cleaners, cosmetics, UV light, temperature, lens dehydration etc. can also be tested. The following chapter deals with initial investigations performed to date using this *in vitro* ocular spoilation model.

CHAPTER 7.

Examples of the use of the *in vitro* model - materials reaction.

7.1. Introduction.

A simple working *in vitro* model system which mimics the interaction between synthetic contact lens materials and the biological environment in the eye, leading to ocular spoilation was developed as described in the previous chapter. This *in vitro* model system in conjunction with the developed analytical techniques was then available to investigate some aspects of mechanisms of tear-lens interaction .

This *in vitro* model provides an ideal system for analysing the interaction between the biological interfacial conversion process and extrinsic factors, such as lens dehydration, cleaning regimes, lens handling, cosmetics etc.. In addition to these extrinsic factors the *in vitro* system allows the study of novel hydrogel materials, hard contact lens materials, the effect of wear schedule and the effect of the particular components of the tear fluid (for example proteins such as lysozyme and lipid fractions such as fatty acids, cholesterol esters etc.), by altering their concentration in the surrogate tear fluid. The *in vitro* model also removes complicating factors such as variation between patient's tear chemistry, lens handling technique etc. and allows strict control over the conditions under which the investigations are carried out.

The initial studies performed were based on the effect of the contact lens composition both hydrophilic or hard; the effect of daily or extended wear schedules and the effect of the composition of the tear fluid on spoilation. All the models used 1:2 (v/v) solution of foetal calf serum (FCS) diluted with phosphate buffered saline (PBS) (pH 7.4) as a tear substitute. The surrogate tear fluid was modified by spiking with different combinations of 0.1g/25ml each of lysozyme, albumin and human-γ–globulins. The

shaker model involves placing a number of small glass beads in a vial, in order to provide an uneven surface on which the contact lenses sit. This will allow contact with the air and surrogate tear solution, the latter being pipetted into the vial to a level just below the 'upper surface' of the glass beads. The prepared vials are then placed on a shaker. This was to enhance the air and tear contact with the lense. The tear solution in the vials was replaced every 24 hours to maintain a supply of undenatured protein and lipid components.

The 'drop and dry' model involves the lenses being placed on dome supports on labelled glass slides and 100µl of the surrogate tear solution being dropped onto the surface every 24 hours.

Prior to their exposure to the surrogate tear solution the fluorescence spectra of the lenses were recorded. Their fluorescence spectra were also recorded every three days, after the lenses had been rinsed to remove any 'dried' on surrogate tear solution which was not bound to the hydrogel surface. These fluorescence spectra were recorded at an excitation wavelength of 360nm, as the emission of adsorbed tear lipid components on the lens surface is optimal at around this excitation wavelength.

7.2. Results.

7.2.1. Investigation 1: The effect of introducing a negative charge to the hydrogel composition.

Many lens materials are based on high (~70%) equilibrium water content (E.W.C) copolymers of vinyl pyrrolidone and methyl methacrylate. Claims are frequently made that 'ionic' lenses are more susceptible to spoilation, although the surface of blood vessels carry

a negative charge. These experiments were designed to see if the introduction of small quantities of 'ionic' methacrylic acid produced changes in an otherwise uncharged hydrogel. A series of vinyl pyrrolidone and methyl methacrylate copolymer contact lenses (E.W.C. 70%) were used in this study. The Control lens was of an unmodified copolymer of vinyl pyrrolidone and methyl methacrylate. This composition was modified to include 0.1, 0.2 and 0.5% methacrylic acid. These lenses were designated MA01, MA02 and MA05 respectively. Four lenses of each type were used in the shaker model and two of each type in the drop and dry model. The values shown in the histograms represent the average values with a reliability of $\pm$10%. The lenses were exposed to the surrogate tear fluid on a daily wear basis for 21 days.

The overall pattern of spoilation can be summarised as follows:-

Control > MA05 > MA02 > MA01. The pattern of spoilation which is observed with the fluorescence intensity values can also be observed under the optical microscope. Initially the lenses show no deposited material or artifacts of manufacture. After exposure to the surrogate tear solution the manifestations of spoilation are observed, to varying degrees, on the lenses.

All the lenses show the ability to attract surrogate tear fluid components. The greatest degree of spoilation in each case occurs with unmodified vinyl pyrrolidone and methyl methacrylate copolymer. The modified lenses containing methacrylic acid follow the the pattern MA05 > MA02 > MA01.

The difference in spoilation between the unmodified copolymer (Control) lenses and the lenses modified with methacrylic acid is quite large. The difference between the

individual modified lenses is, however, smaller. This trend is observed with both forms of the model (figures 7.1, 7.2). By taking an overall average value of all six lenses from both models the effect of the addition of the methacrylic acid is clearly visible (figure 7.3). These results show that the addition of a small amount of negative charge in the form of methacrylic acid does reduce the degree of spoilation. If however a large dose of negative charge is added (MA05) the trend is reversed and spoilation is enhanced relative to the lower levels of methacrylic acid incorporated.

Figure 7.1:- Effect of methacrylic acid modification on *in vitro* spoilation.

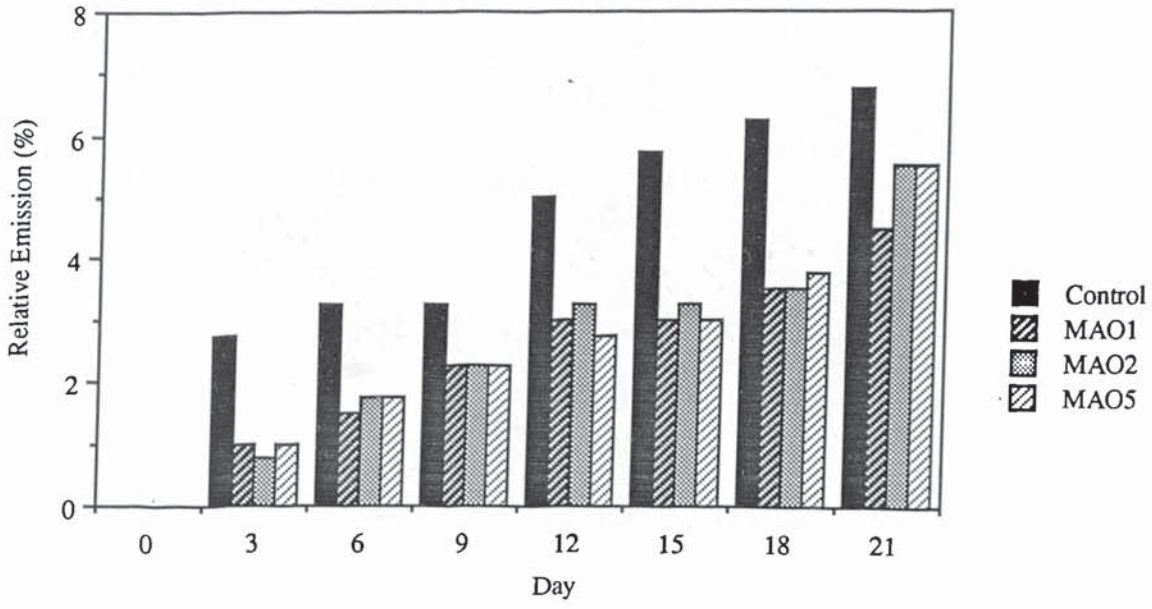


Figure 7.2:- Effect of methacrylic acid modification on *in vitro* spoilation using the drop and dry model.

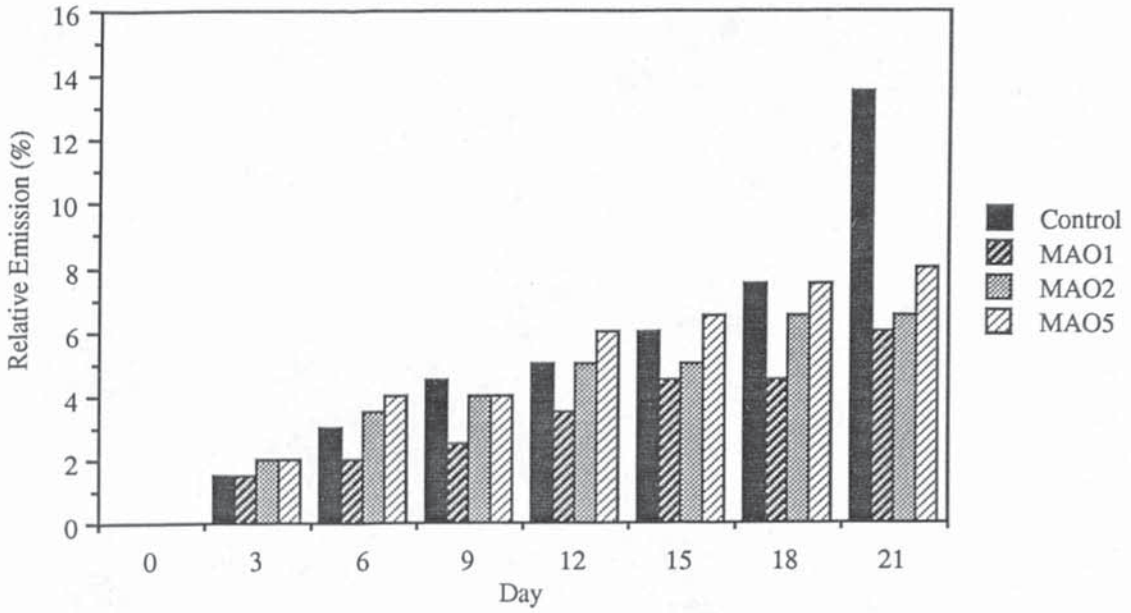
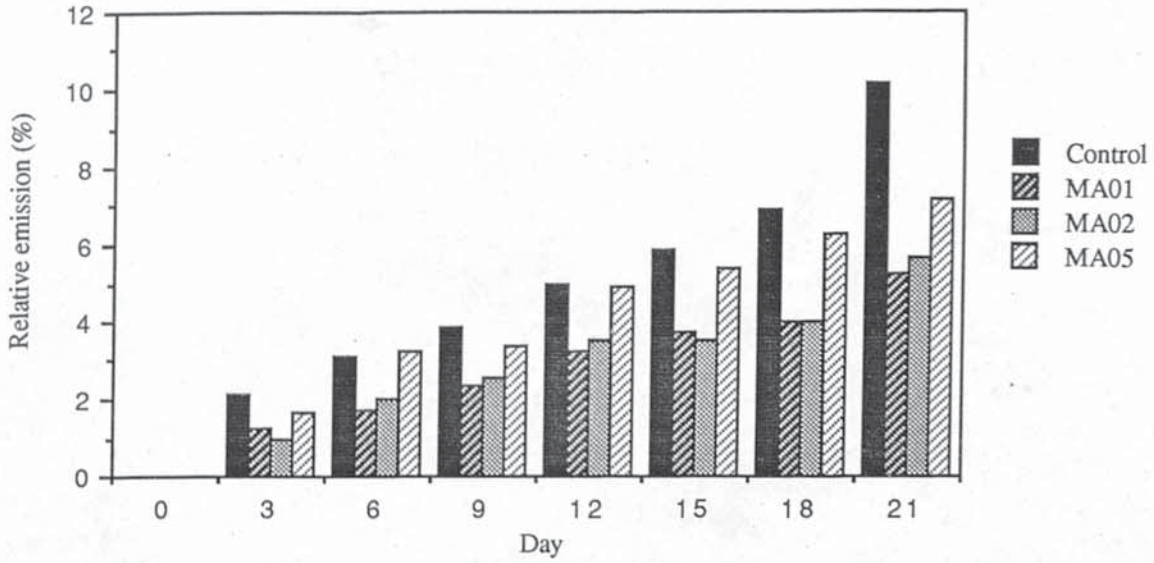


Figure 7.3:- Overall pattern of *in vitro* spoilation of methacrylic acid modified lenses.



7.2.2. Investigation 2: The effect of a negative charge on protein - enhanced surrogate tear fluid.

In order to further investigate the results obtained by introducing a negative charge to the hydrogel structure the effect of FCS spiked with tear proteins was investigated. Only the shaker model was used for this investigation. Two types of surrogate tear solution were used; FCS spiked with 0.1g/25ml lysozyme and FCS spiked with 0.1g/25ml albumin and 0.1g/25ml human-γ-globulins. The lenses were exposed to the modified surrogate tear fluid on an extended wear model basis for 21 days.

The overall pattern of spoilation alters as the lenses build-up a deposited layer. All the lenses show the ability to attract the tear components from the surrogate tear solution. The addition of lysozyme to the surrogate tear solution demonstrated the attraction of the protein to the negatively charged methacrylic acid (figure 7.4). The initial stages of spoilation, however, suggested that the MAO2 lenses attracted the greatest quantity of tear components. This may be due to the spatial distribution and 'blocking' of the negative charge so a gradual decrease is then observed in the rate of spoilation of these lenses. The (MAO5) lenses have a greater concentration of negative charge which has attracted a greater quantity of deposited components after 21 days exposure to the surrogate tear solution spiked with lysozyme.

The addition of albumin and human-γ-globulins to the surrogate tear solution shows a greater variation in the quantity of material attracted to the different lens types (figure 7.5). After 21 days the Control and MAO1 lenses seemed to have attracted the greatest quantity of tear components and the MAO2 dosed lenses had attracted the least.

The initial build-up of the protein was greater on the methacrylic acid dosed lenses than the unmodified copolymer. After 12 days however this trend had reversed with the greatest degree of spoilation being demonstrated by the unmodified copolymer and low dosed MAO1 lenses. The reason for these trends is unclear, but may be due to the influence of the the initial deposited layer on subsequent deposition. It is apparent, however, that the presence of positively charged lysozyme causes an enhanced deposition on negatively charged lenses.

Figure 7.4:- Effect of methacrylic acid modification on in vitro spoilation with lysozyme.

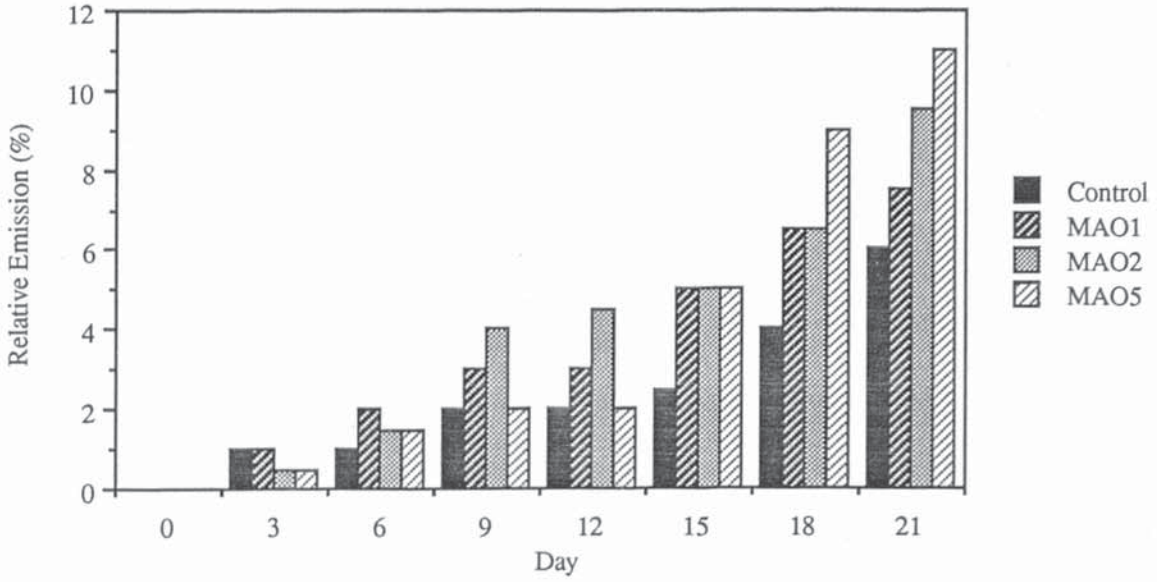
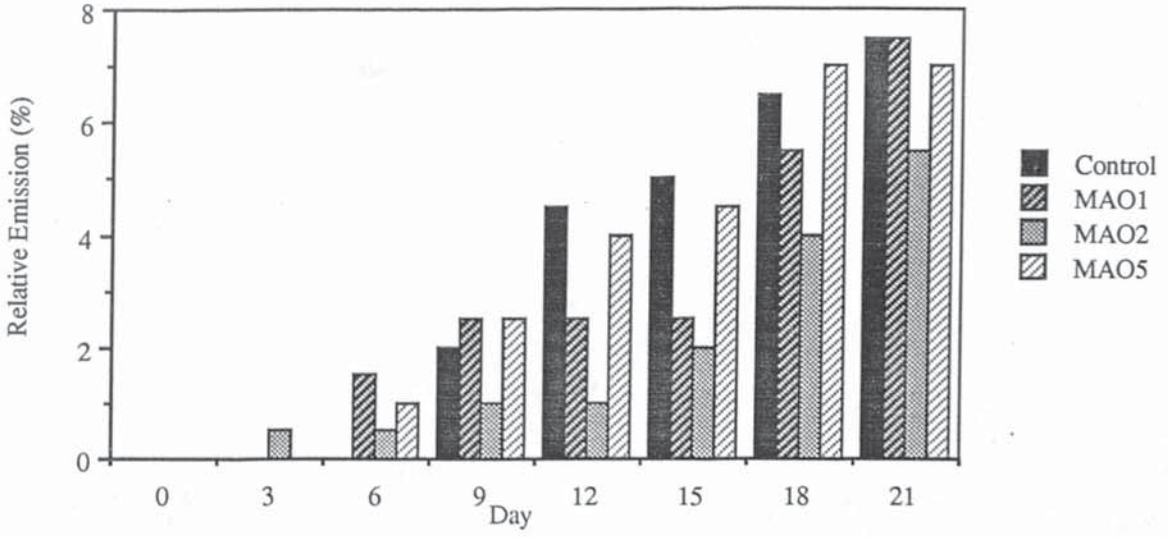


Figure 7.5:- Effect of methacrylic acid modification on in vitro spoilation with albumin and globulins.



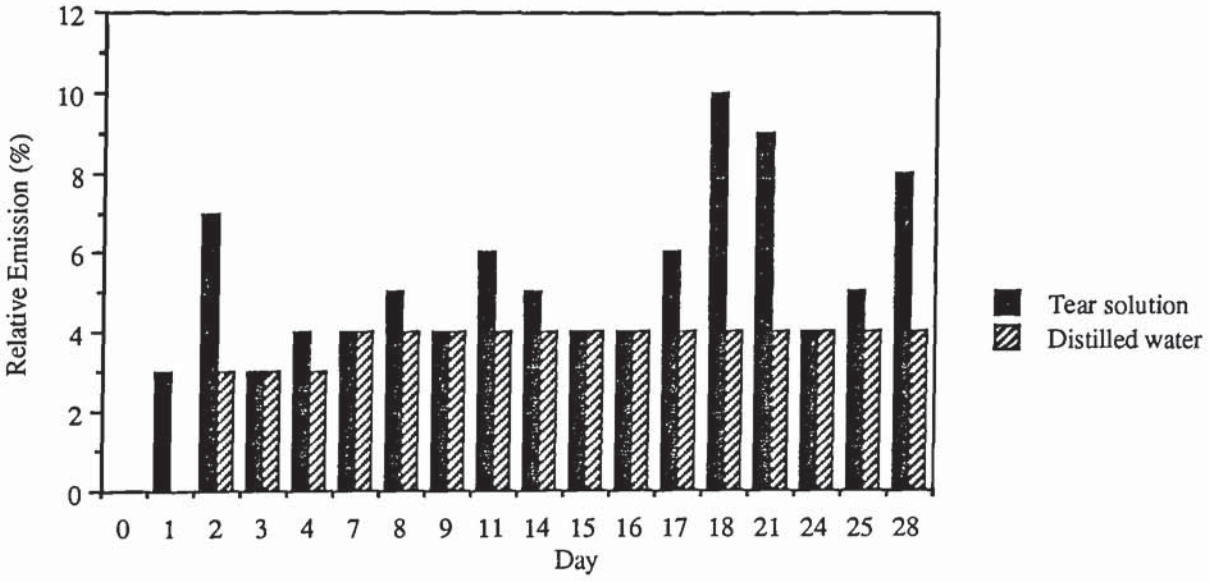
7.2.3. Investigation 3 The effect of hard lens composition.

Five hard lenses were tested. These lenses contained varying amounts of trifluoroethylmethacrylates and methacrylic acid. The lenses were exposed to the surrogate tear fluid on a daily wear model basis using the shaker model. The lenses were stored in distilled water overnight. Fluorescence spectra were recorded (a) after removal from the tear model with a single rinse and (b) after the overnight period in distilled water. These results are shown as histograms.

The results of the preliminary spoilation studies using hard lens materials indicate that these materials are also capable of attracting the tear components from the surrogate tear solution. For all the samples tested a build-up of material followed by a lower fluorescence intensity value is observed. The build-up of the tear components reaches a constant value in each case after overnight storage. A variable quantity of tear components are however attracted over the wear period of a day. This is believed to be due to the drying of the tear components onto the surface of the lens, but not actually binding to the surface. As a result the lens builds up a large quantity of tear components which then break away from the lens surface as they are not actually bound to it.

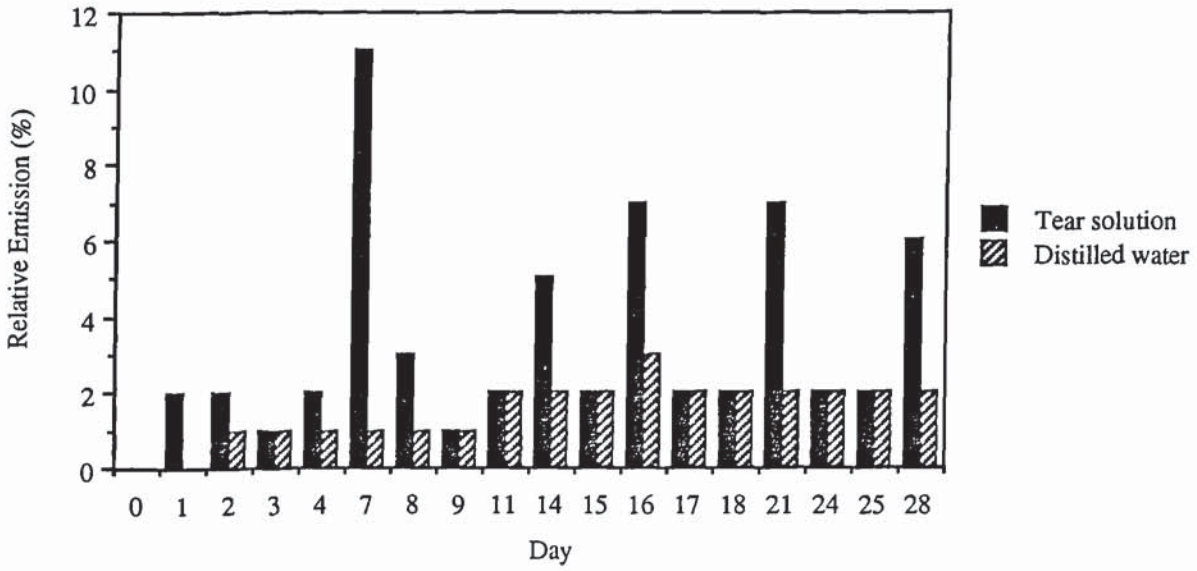
Hard lens sample 1 shows a variable build-up of tear components on it's surface (figure 7.6). After 7 days the relative fluorescence intensity does not fall below 4 units after being stored overnight in distilled water. There is a variable amount of tear component deposited on the lens surface over the wear period of a day, which increases towards the end of the investigation.

Figure 7.6:- of *in vitro* spoilation on hard lens sample 1.



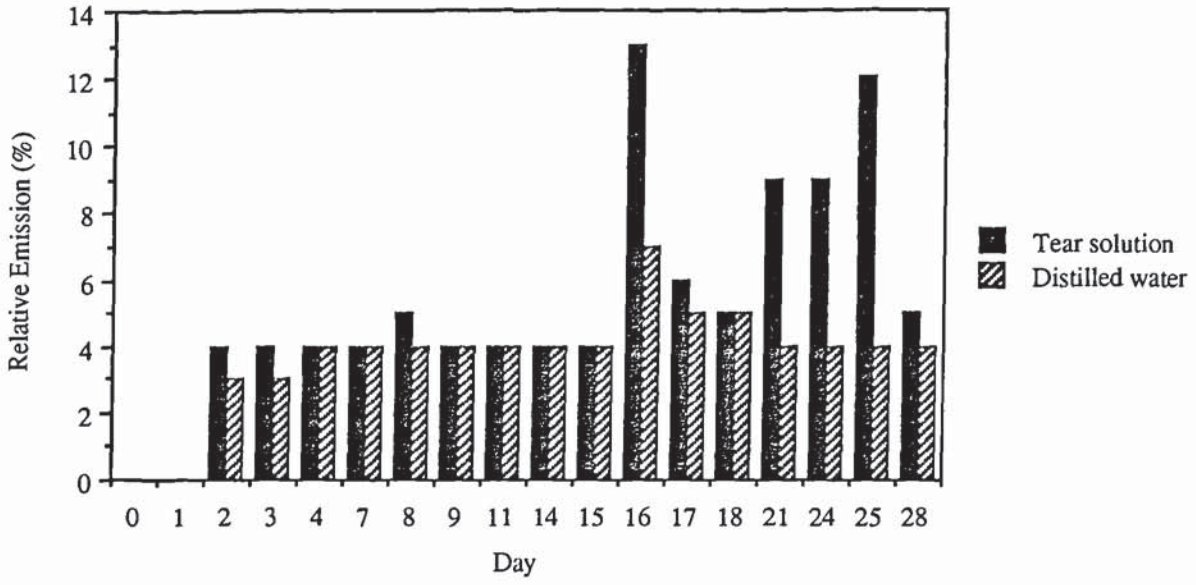
Hard lens sample 2 shows a variable attraction of tear components over the day during the investigation (figure 7.7). Variable quantities of tear components are deposited over the wear period of a day. The fluorescence intensity after the overnight storage period falls to a constant value of around 2 units after 11 days simulated wear.

Figure 7.7:- Effect of *in vitro* spoilation on hard lens sample 2.



Hard lens sample 3 shows a gradual build-up of deposited tear components after daily exposure to the surrogate tear solution (figure 7.8). This deposited tear film reaches a constant level of around 4 units after 4 days. These lenses did however show a greater post-storage value between days 16-18, before returning to around 4 units. This is probably due to the build-up of non-bound components which are then lost from the lens surface.

Figure 7.8:- Effect of *in vitro* spoilation on hard lens sample 3.



Hard lens sample 4 shows the lowest initial fluorescence value after storage, at around 1 unit until day 24, where the value increases to around 4 units (figure 7.9). This lens material did however attract a greater quantity of tear components than the other lens materials over the course of a day.

Hard lens sample 5 shows an almost immediate degree of spoilation. After 2 days the lens had a constant value of around 1 unit which was not increased after the storage period for the 28 days of the study (figure 7.10). The variable attraction of tear components was also observed to a lesser extent than with the other hard lens materials.

Figure 7.9:- Effect of *in vitro* spoilation on hard lens sample 4.

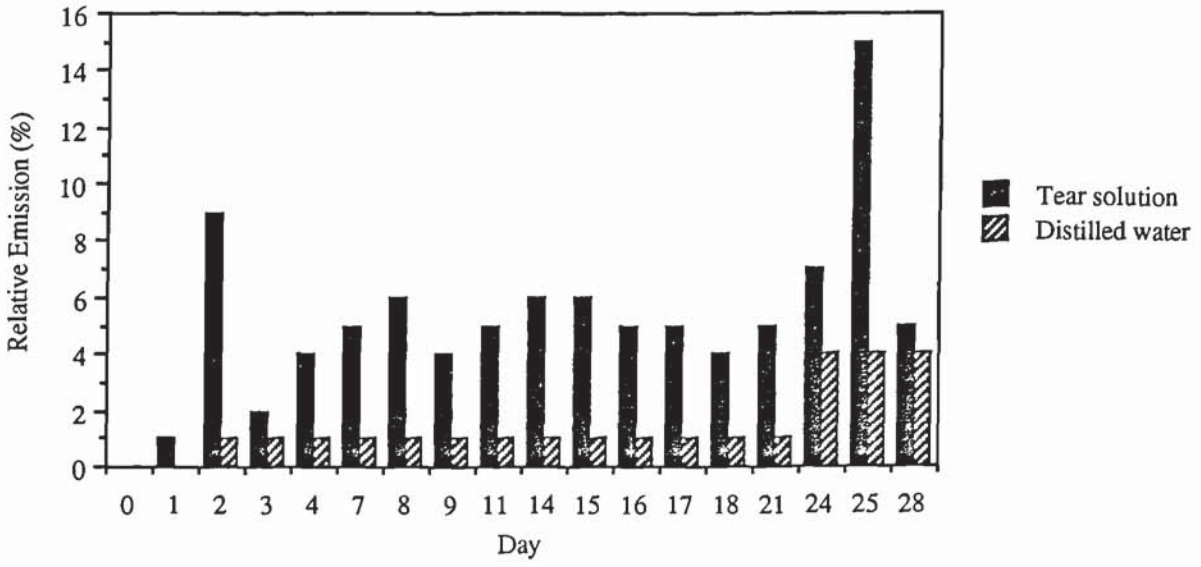
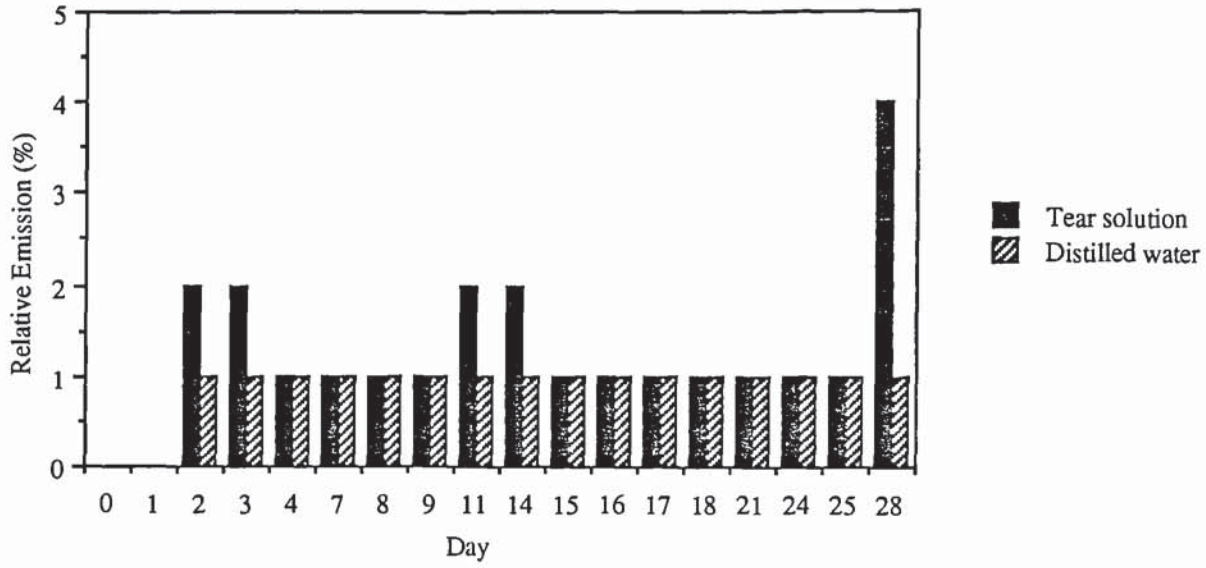


Figure 7.10:- Effect of *in vitro* spoilation on hard lens sample 5.



7.3. Discussion of the results.

All the investigations show that the lenses are capable of attracting tear components from the surrogate tear solution and therefore mimicking *in vivo* spoilation *in vitro*. The effect of introducing extreme types of materials into the contact lens can be easily observed e.g. methacrylic acid. It is also possible to show the effect of these materials on an altered tear composition which shows that under certain circumstances the progression of spoilation may be altered, although the initial events are believed to be the same.

These initial investigations were designed to show that it is possible to study the effects of spoilation on hard lens materials as well as hydrophilic ones using this *in vitro* model. Although the variation in spoilation may reasonably be labelled as dependent upon the varying proportions of methacrylic acid and trifluoroethylmethacrylates in the lens materials. The precise compositions were not available at the time of study. To avoid pointless speculation no detailed conclusions between supposed spoilation behaviour are alleged.

These initial studies do not however, take into account the effect of extrinsic factors such as cleaning which is investigated in the following studies described in chapter 8.

CHAPTER 8.

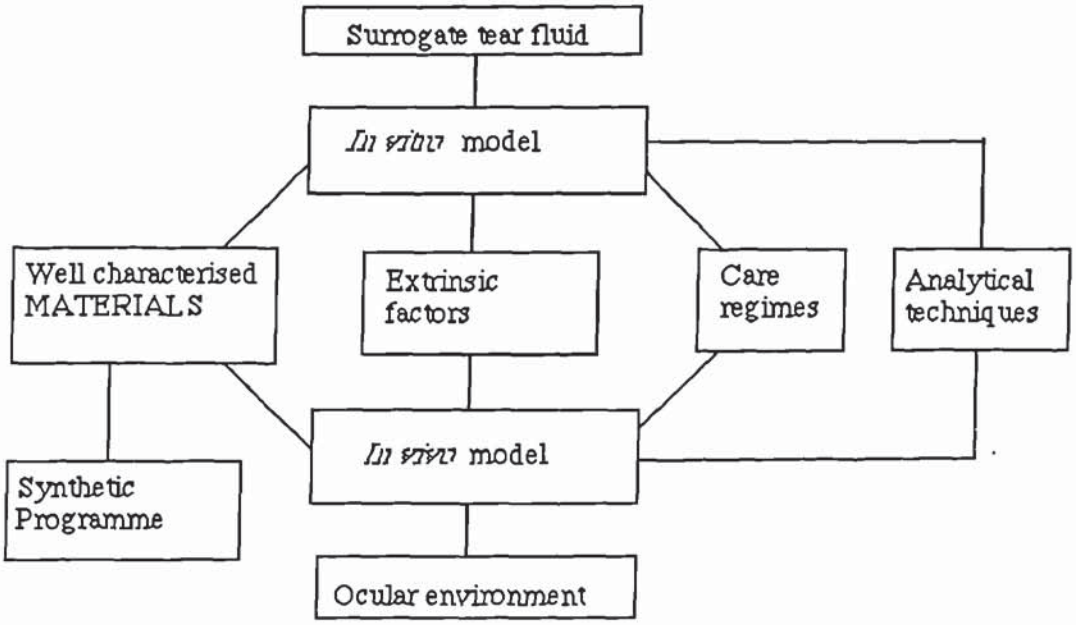
In vitro investigation of some extrinsic factors involved in ocular spoilation.

8.1. Introduction.

A number of extrinsic factors which contribute to lens spoilage are cited in the literature [10]. These include cosmetics, poor lens handling, airborne foreign bodies, dust, toxic fumes and metal particles. The cleaning regime also effects lens spoilage. Earlier in this thesis a question was raised as to whether the cleaning regimes are effective or whether an insoluble process capable of occurring within a day is involved in the initial events of interfacial conversion.

The involvement of extrinsic factors in the overall pattern of ocular spoilation can be summarised in figure 8.1.

Figure 8.1:- Involvement of extrinsic factors in ocular spoilation.



In order to investigate the involvement of cleaning regimes in the spoilation process two types of regime were investigated using the *in vitro* model system. The two types of

care regime were heat sterilisation and enzyme cleaners.

Both investigations used the shaker model. The glass beads were placed in the vials in order to provide an uneven surface on which the lenses would sit. This allows contact with the air and surrogate tear fluid, the latter being pipetted into the vials to a level just below the 'upper surface' of the glass beads. The prepared vials were then placed on a shaker. This was to enhance the air and tear contact with the lenses. The surrogate tear solution was replaced every 24 hours to maintain the supply of undenatured protein and lipid components.

8.2. Results.

8.2.1. Investigation 1: The effect of heat sterilisation of the in vitro spoilation of three low water content polyHEMA based soft contact lenses.

Four different surrogate tear solutions were used during this investigation:-

1. FCS diluted 1:2 (v/v) with PBS.
2. FCS diluted 1:2 (v/v) with PBS and a solution of 0.5g/25ml lysozyme used alternately.
3. FCS diluted 1:2 (v/v) with PBS and spiked with 0.5g/25ml of lysozyme, albumin and cholesterol.
4. FCS diluted 1:2 (v/v) with PBS and spiked with 0.5g/25ml of albumin, lysozyme, human-γ-globulins, cholesterol and linoleic acid.

Three polyHEMA based soft contact lenses; one lathe cut and two cast polymerised which had been made from different source monomers were analysed. All lenses were produced by commercial manufacturers. There may be a variation in the processes of

manufacture which could produce an overriding property capable of altering the progression of spoilation. The aim was to test the effect of thermal sterilisation on these three lenses which although they were specially produced were representative of the commercial polymerisation technology and monomers available. These lenses were polyHEMA lenses (A) and (B) and tetrafilcon-A (C). Lens A had been lathe cut; and lenses B and C cast polymerised.

For each solution two lenses were heat sterilised and one lens, a Control, was not; also each lens was subjected to wearing simulation cycles as follows:-

1. Agitate the lens briefly in surrogate tear solution for 1.5 hours.
2. Rinse briefly with Bausch and Lomb saline preserved with sorbic acid to remove excess surrogate tear solution which is not bound to the lens surface.
3. Disinfect the lenses in Bausch and Lomb saline preserved with sorbic acid using a Bausch and Lomb heat sterilisation kit and
4. Soak lenses in fresh Bausch and Lomb saline preserved with sorbic acid for 45 minutes (sleep period).

This cycle was repeated three times daily. The lenses were examined with an optical microscope under phase contrast illumination and their fluorescence spectra recorded prior to their subjection to the *in vitro* model and prior to being soaked overnight in Bausch and Lomb saline preserved with sorbic acid.

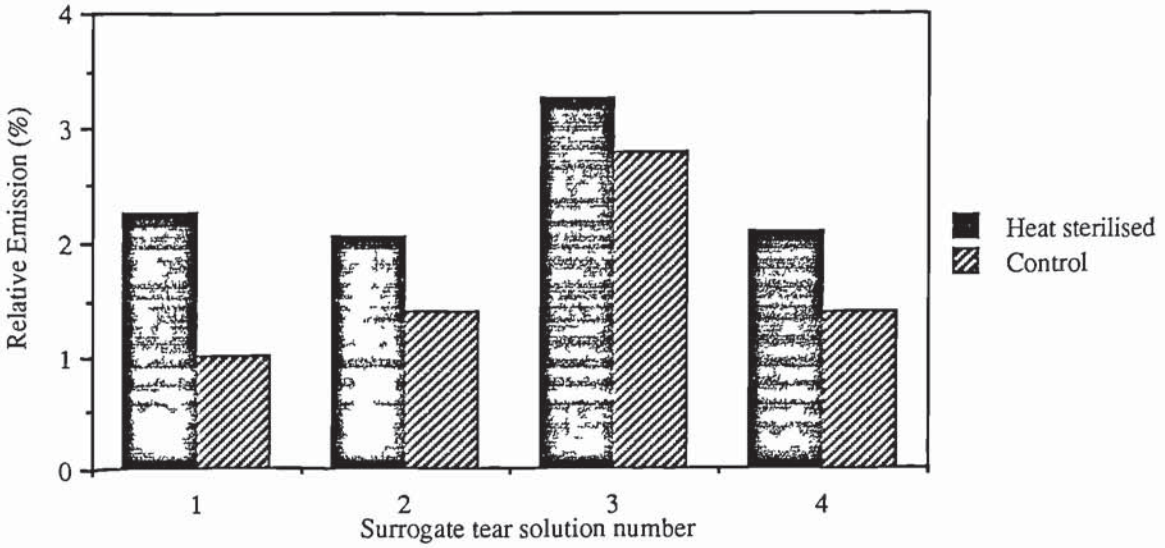
The base values of the unspoilt lenses fluorescence intensity values are A 3.0, B 3.6 and C 3.7. The fluorescence intensity values of the spoilt lenses, after 7 days

spoilation, shown represent the relative emission which is the final fluorescence intensity minus the initial fluorescence intensity.

All the lenses show an increase in their fluorescence intensity as compared to their unspoilt state. The heat sterilised lenses show a greater increase in their fluorescence intensity than the non-heat sterilised lenses.

The lathe cut polyHEMA lenses, (A) show their greatest increase in intensity after spoilation with surrogate tear solution 3 (spiked with albumin, lysozyme and cholesterol). The increase in the fluorescence intensity obtained with surrogate tear solution 1, surrogate tear solution 2 and surrogate tear solution 4 are similar. There is considerably less spoilation in the non-heat sterilised lens, an effect most marked with surrogate tear solution 1 (figure 8.2). This tear solution is the only one that does not contain lysozyme.

Figure 8.2:- Effect of heat sterilisation on the lathe cut polyHEMA lenses (A)



The cast polymerised polyHEMA lenses (B), also show their greatest increase in fluorescence intensity after spoilation with surrogate tear solution 3 (spiked with albumin, lysozyme and cholesterol). There was a gradual decrease in fluorescence intensity produced by spoilation with surrogate tear solution 1, surrogate tear solution 4 and surrogate tear solution 2. The Control lenses fluorescence intensity values decrease in sequence surrogate tear solutions 2, 4 and 1. Again the non-lysozyme containing solution in conjunction with no heat sterilisation produces the lowest degree of spoilation. Interestingly in the non-thermally sterilised cases the cast polymerised polyHEMA B lenses showed a lower degree of spoilation than the lathe cut lenses (A). Heat sterilisation produced a unifying effect however and the values for the four solutions show little difference.

Figure 8.3:- Effect of heat sterilisation on the cast polymerised polyHEMA lenses,B.

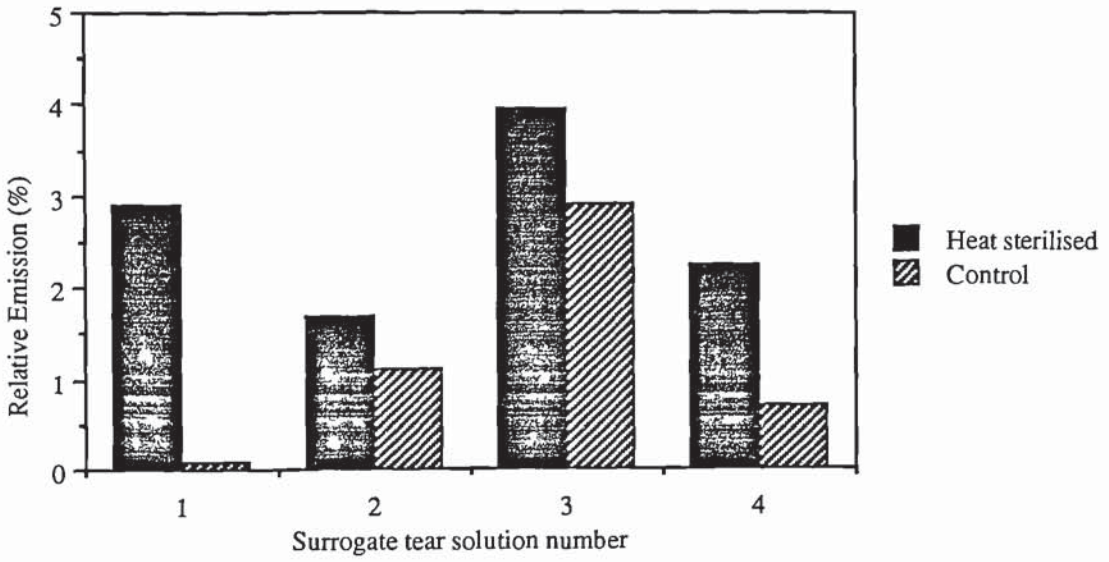
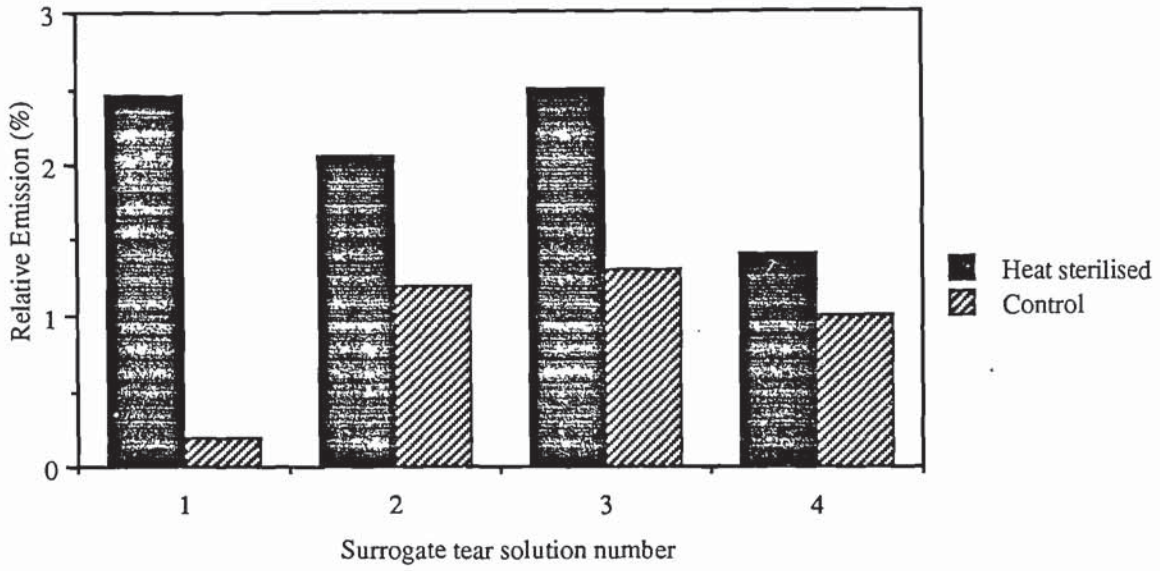


Figure 8.4:- Effect of heat sterilisation on the cast polymerised tetrafilcon-A lenses, C.



It is interesting that the tetrafilcon-A lenses (C), in the case of the heat sterilisation protocol, show levels of accumulation that are lower than or similar to that shown by lenses A and B. The difference is not great, but appears to be constant. It is, perhaps, significant that in the cases of solutions 3 and 4 which are most representative of tears lens C behaves measurably better than lenses A and B. In the case of the non-heat sterilsed protocol both the cast polymerised lenses C and B behave measurably better than the lathe cut lens A. It is, however, reassuring that there was no dramatic (i.e. tenfold) difference between the different materials.

Optical microscopy gave a useful qualitative picture of the spoilation. The heat sterilised lenses show patches of transparent film, under phase contrast, with numerous small deposits. The non-heat sterilised lenses show less transparent film areas, under phase contrast, and fewer deposits. The heat sterilised lenses spoilt with surrogate tear solution 3 (spiked with albumin, lysozyme and cholesterol) show extensive transparent film areas, under phase contrast,with numerous deposits. There is also quite a high degree of spoilation observed on non-heat sterilised lenses.

The cast polymerised polyHEMA lenses (B) show a decrease in the degree of spoilation dependent upon on the medium used. The greatest degree of spoilation, observed as a patchy transparent film with numerous small deposits, was obtained with surrogate tear solution 3 (spiked with albumin, lysozyme and cholesterol). Similar artifacts with a gradual decrease in frequency are observed with the media as follows surrogate tear solution 1, surrogate tear solution 4 (spiked with albumin, lysozyme, human-γ-globulins, cholesterol and linoleic acid) and surrogate tear solution 2 (diluted FCS and a solution of

lysozyme used alternately). The non-heat sterilised lenses show only a few spoilation artifacts except for the lens spoilt in surrogate tear solution 3 (spiked with albumin, lysozyme and cholesterol).

The cast polymerised tetrafilcon-A lenses (C) show a gradual decrease in the artifacts of spoilation over the four week trial period. The greatest degree of spoilation being obtained with surrogate tear solution 1 and the least with surrogate tear solution 4 (spiked with albumin, lysozyme, human-γ-globulins, cholesterol and linoleic acid). The non-heat sterilised lenses show less spoilation than the heat sterilised lenses, particularly the Control lens spoilt with surrogate tear solution 1.

All heat sterilised lenses exhibited some degree of fluorescent material when examined under fluorescent illumination. The extent of these fluorescent areas being determined by the degree of spoilation of the lenses.

In summary, all lenses show the ability to attract tear components. cast polymerised tetrafilcon-A lenses (C) spoil less readily than the other lenses and appear to be more susceptible to lipid than protein spoilation. The lathe cut polyHEMA lenses (A) appear to show greater lysozyme sensitivity than do cast polymerised polyHEMA lenses (B) although both seem prone to cholesterol contamination. Having made these observations it must be said that the overall difference between the behaviour of lathe cut polyHEMA lenses (A) and cast polymerised polyHEMA lenses (B) is slight, rather than dramatic.

These observations may however be affected by the before and after methodology

used, rather than monitored analysis over a number of days and the use of a Perkin-Elmer spectrophotofluorimeter.

Elsewhere within the research group different studies on protein layers on contact lenses have shown that protein fragments remain on the lens surface after cleaning and that the specificity of the enzymes for different proteins is variable e.g. lactoferrin. As a result two enzyme cleaners were examined to observe the possible consequences of accumulation of deposited protein fragments etc. on ocular spoilation. The preparations chosen contain a single protease (Hydrocare Fizzy) and a dual enyzyme - protease and lipase (Clen-zym).

8.2.2. Investigation 2. To determine the efficacy of two enzyme cleaners.

The surrogate tear solution used in this investigation was 1:2 (v/v) FCS diluted with PBS. The contact lenses used in this investigation were polyHEMA lenses. Four lenses were used for testing the cleaning regime and two of each type as Controls.

The model was run for a total of 28 days on a daily basis. Prior to their exposure to the tear solution the fluorescence spectra of the lenses were recorded. The following protocol was then followed;-

(i) Run fluorescence spectra of the lenses.

(ii) Sterilise the lenses prior to use to prevent any microbial contamination of the tear solution which, leads to degradation.

(iii) Agitate the lenses in protein and lipid enriched artificial tear solution for 14 hours.

(iv) Rinse briefly in saline solution, to remove excess diluted foetal calf serum

(v) Run fluorescence spectra (equals 'tear solution')

(vi) Treat the lenses chemically using Clen-zym or Hydrocare Fizzy for a two hour soaking period. The Control lenses were placed in saline for this two hour period.

(vii) Run fluorescence spectra (equals 'cleaned').

(viii) Soak lenses in soaking solution 'overnight'.

(ix) Run fluorescence spectra (equals 'distilled water').

The fluorescence spectra were recorded at an excitation wavelength of 360nm. The emission peaks from adsorbed tear model components on the lens surface occur around a wavelength of 480 nm. The relative transmission at this wavelength is used to convert the large number of spectra obtained into histograms which summerise the data. The relative transmission of an unspoiled lens is set at 0 in these histograms.

The protocol described above was used to study the effect of both daily application and weekly application of the two enzyme cleaners.

Figure 8.5 shows the effect of daily application of Clen-zym and should be compared with the Control experiments shown in figure 8.6 where the same soaking periods were used but the enzyme cleaning step was replaced by a "blank" (saline solution containing no enzyme). Comparison of the drop in relative intensity of the transmission between the spoilation (solid black bar) and enzyme cleaning step (hatched bar) in figure 8.5 shows the effect of the enzyme whereas the blank experiment generally shows no drop.

Figure 8.5:- *In vitro* spoilation and daily Clen-zym cleaning regime.

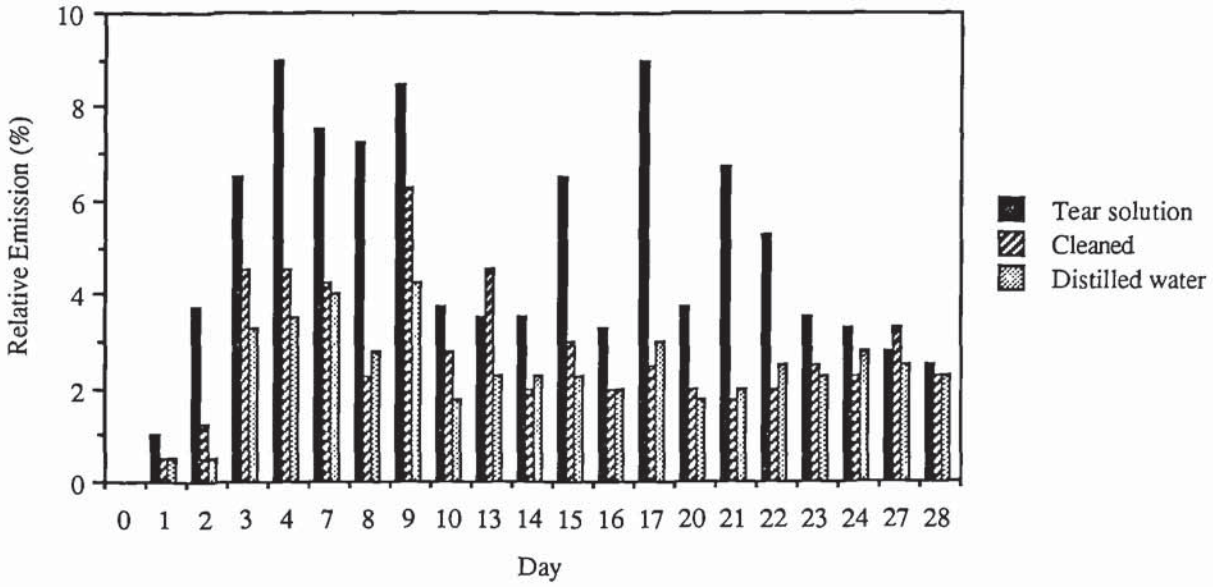
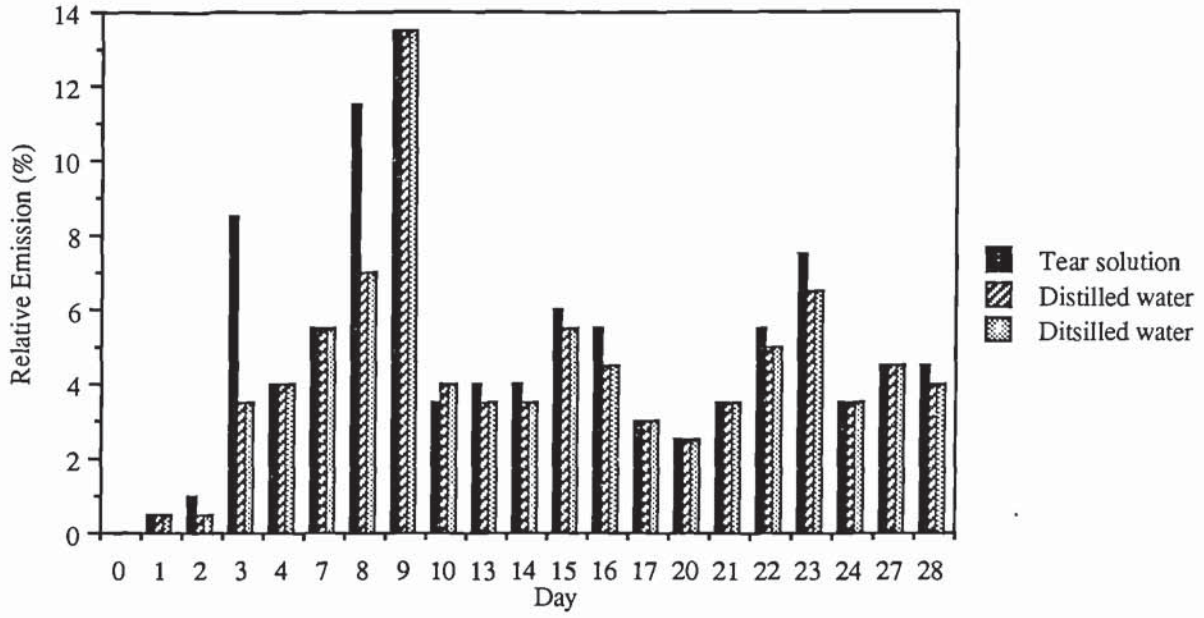


Figure 8.6:- *In vitro* spoilation model - Control lenses for Clen-zym regime.



It is a function of the experimental conditions used here (no digital cleaning and only modest agitation during cleaning) that a substantial deposited layer builds up within 5-10 days on the clean lens. Physical loss from this layer then occurs and the deposition process achieves a pseudo-equilibrium with the primary adsorbed layer. This is very similar to the way build up occurs in the eye.

Comparison of figures 8.7 and 8.8 shows the effectiveness of Hydrocare cleaning relative to the 'blank' experiments that were performed alongside. It should be noted that the results plotted are the average of several individual experiments, as noted in the experimental protocol. Two points emerged as the experiments were carried out and accumulated.

Figure 8.7:- ***In vitro*** **spoilation and daily Hydrocare cleaning regime.**

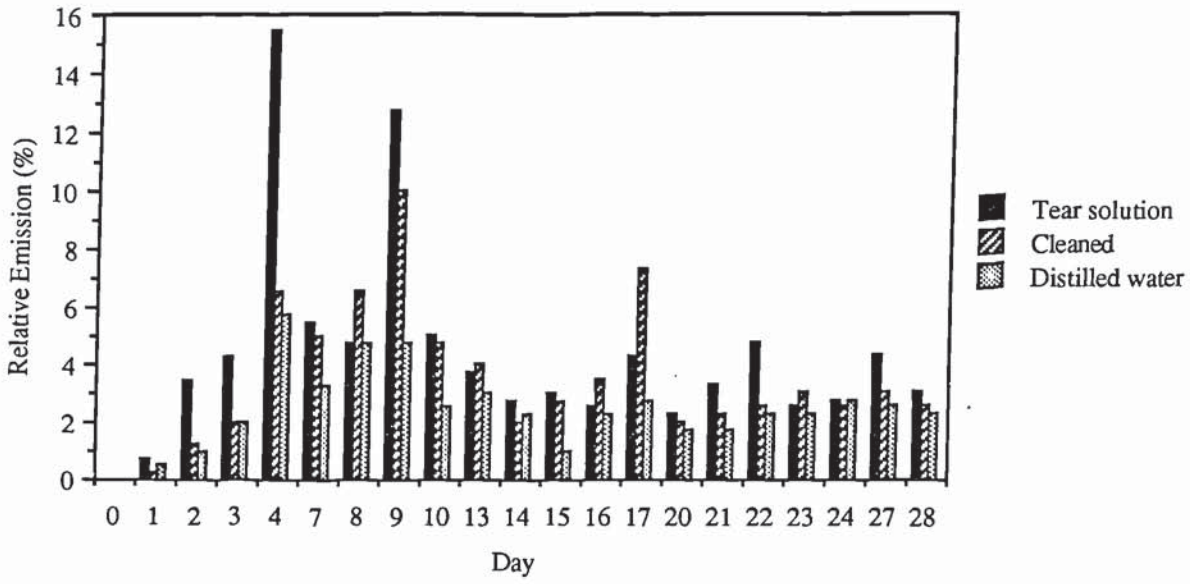
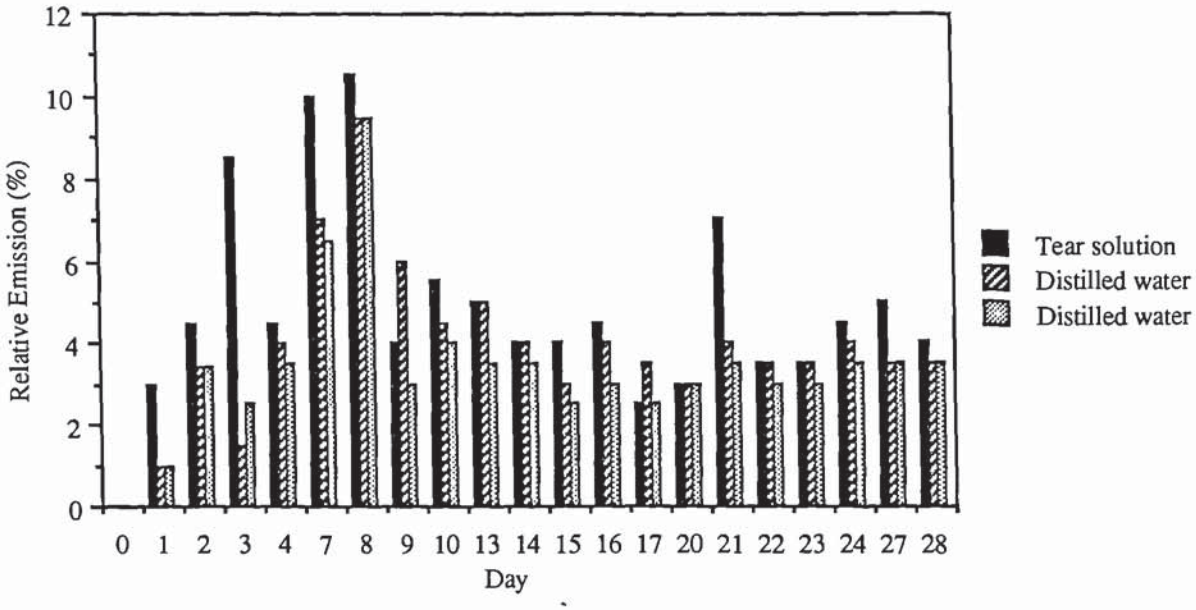


Figure 8.8:- *In vitro* spoilation model - Control lenses for Hydrocare regime.



The first is that the Clen-zym results showed less scatter than did the Hydrocare results. The second is that for the first 10 day period the nett difference between the spoiled lens (solid bar) and cleaned lens (hatched bar) was greater for Clen-zym experiments than for Hydrocare experiments. Against this must be set the facts (a) that the Hydrocare-cleaned lenses, and (b) that the long term spoilation experiments (> 10 days) show that there is less spoilation on the Hydrocare (figure 8.7) than the Clen-zym (figure 8.5) system lenses. This latter point can be seen by simply comparing the height of the hatched or dotted bars in the two figures.

The weekly clean experiments (figures, 8.9, 8.10, 8.11) contain far less data since only four applications of cleaner are involved in the 28 day period. It would not be reasonable to draw conclusion from these experiments alone but they do not conflict with

the observations already made, given the differences in the two sets of experiments.

Figure 8.9:- *In vitro* spoilation model - Weekly Clen-zym cleaning regime.

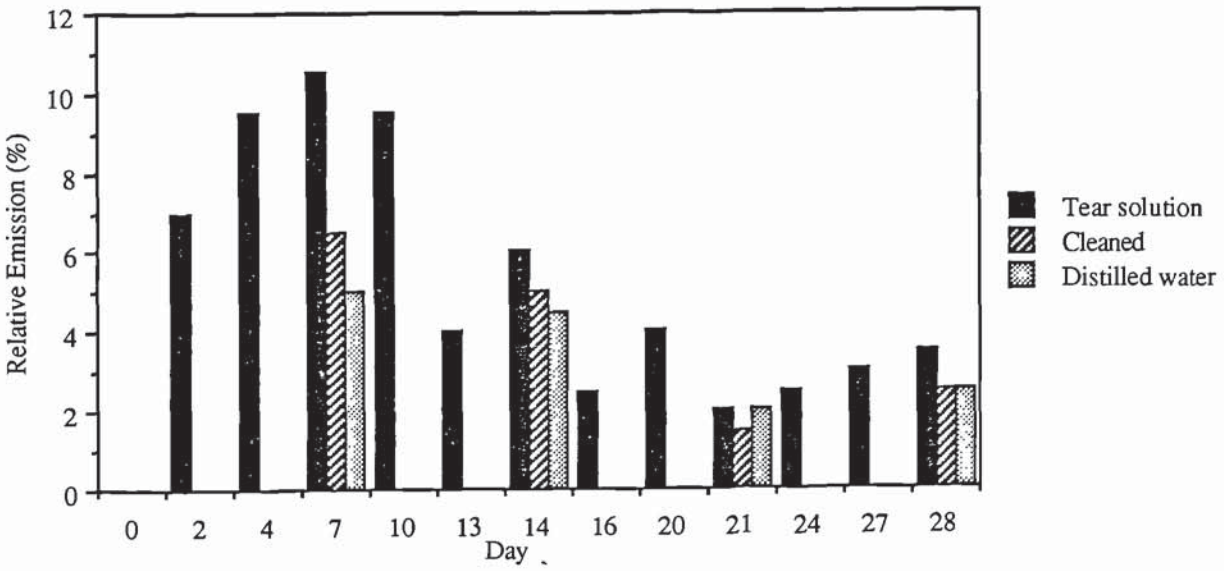


Figure 8.10:- *In vitro* spoilation model- Weekly Hydrocare cleaning regime.

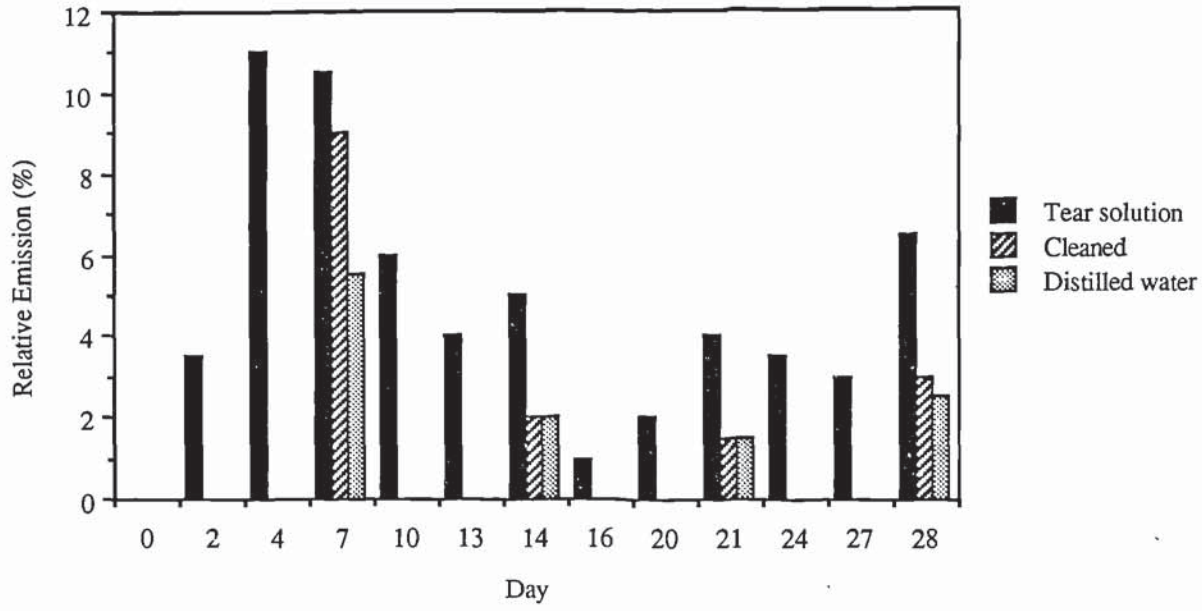


Figure 8.11:- *In vitro* spoilation model - Weekly Control lenses.

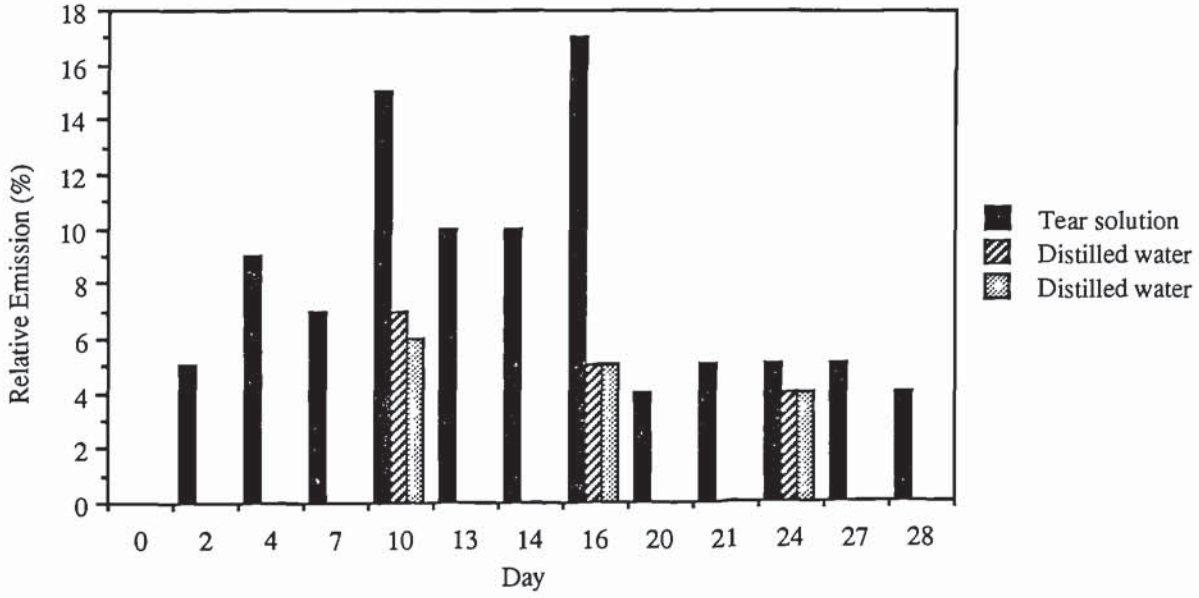
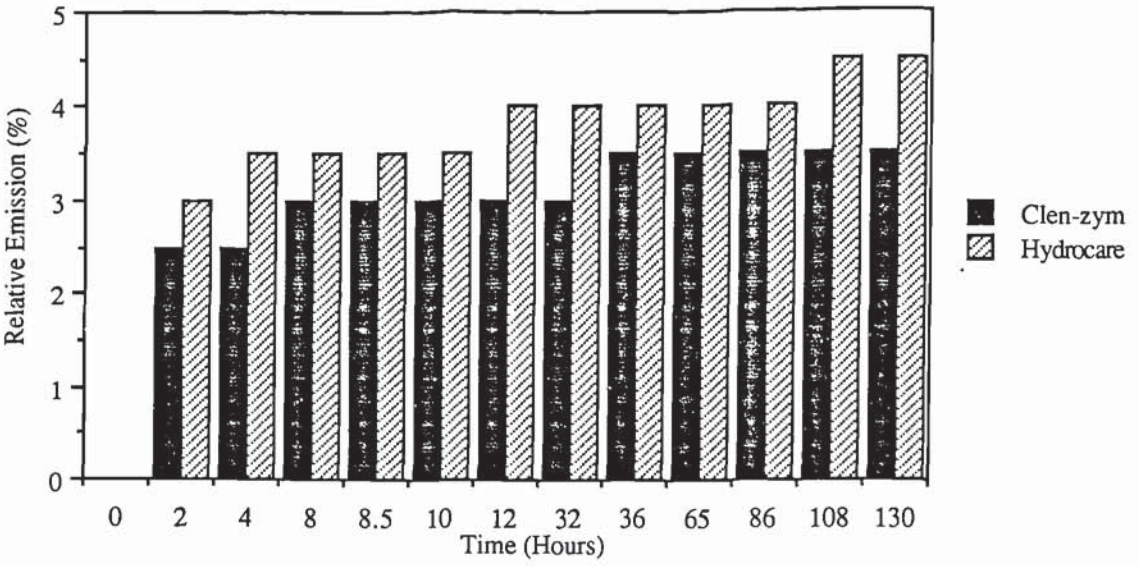


Figure 8.12 shows the relatively rapid build up of enzyme on the surface of clean lenses, in the absence of other biological components. This simply confirms what is well known about molecules of this type.

Figure 8.12:- Enzyme build-up on polyHEMA lenses.



8.3. Concluding discussion.

Both these investigation show that the cleaning regime does have an effect on the build-up on tear components on contact lenses during spoilation.

In investigation 1 all the lenses showed the ability to attract 'tear' components. Cast polymerised tetrafilcon-A lenses (C) spoil less readily than the other lenses and appear to be more susceptible to lipid rather than protein spoilation. The lathe cut polyHEMA lenses (A) appear to show a higher degree of lysozyme sensitivity than the cast polymerised polyHEMA lenses (B) although both seem prone to cholesterol contamination. The overall differences between the behaviour of these two types of lenses is slight rather than dramatic.

In investigation 2 the results can be summarised by figures 8.5-8.11 which show that both Clen-zym and Hydrocare are effective in removal of some proportions of the biological debris deposited from a model tear solution onto contact lenses. Although there are some differences between the systems they are not dissimilar in performance. There is no evidence that Clen-zym removes an appreciable proportion of the lipoidal component.

Indeed, parallel experiments in these laboratories show that any reasonable surfactant - containing soft lens cleaner does the job far more effectively and that enzyme cleaners are only effective if used in conjunction with surfactant cleaners.

Other work in this group, concentrating on the chemistry of spoilation may offer some explanation of the fact that a Clen-zym cleaning step is followed by more deposition

than is a Hydrocare cleaning step. The relative efficiency of the various commercially available enzymes in cleaving tear proteins is quite different. As a result some proteins are left relatively untouched by some commercial enzyme systems whereas others are cleaved into fragments which may still have molecular weight of several thousands. Surprisingly this is not simply a matter of all enzymes of the same broad type (e.g. papain) behaving similarly, since different brands of the same enzyme type behave differently.

CHAPTER 9.

Conclusions and suggestions for further work.

The spoilation phenomena observed on contact lenses and described in the introduction to this thesis, have been attributed to a number of tear components. These include lipids, proteins, mucoproteins, calcium and mucin. However, there was no overall consensus as to the relative importance of these factors in triggering the interfacial conversion processes which cause spoilation in its several manifestations. In particular there was no previous experimental work (largely because of an absence of analytical techniques) to demonstrate the role of lipids in the early stages of contact lens spoilation.

Histological studies showed a number of tear solutes were present in the deposition phenomena and indicated the potential importance of the lipoidal species. Initial studies using agarose gel electrophoresis and thin layer chromatography also indicated the problems involved in analysing the minute quantities of biological debris involved in ocular spoilation. Despite the clinical applications and usage of agarose gel electrophoresis and silver staining, the technique only produced partial resolution of the many tear proteins. As a result another member of the biomaterials research group has developed the SDS-Page electrophoresis system for analysing the tear proteins. Thin layer chromatography (TLC) also produced only partial resolution of the proportion of lipids extracted from a single contact lens. This again was insufficient to allow the analysis of the small variations in tear chemistry to be analysed. Therefore, the need still remained for techniques to analyse the initial stages and minute quantities involved in the interfacial conversion process.

Prior to this study techniques to analyse single contact lenses for lipoidal components were not available, there was a lack of information about the lipids present in individual tear samples and the role of lipids was also unclear. During this study two main

techniques have been developed and utilised in an attempt to understand the initial events involved in the interfacial conversion process. These techniques were particularly concerned with the lipoidal species involved, but also allowed the separate analysis of protein components. The techniques used were high performance liquid chromatography and the use of fluorescence spectrophotofluorimetry. Both of these techniques exhibited the extreme sensitivity required to analyse the deposition process. The high performance liquid chromatography (HPLC) technique enabled both the analysis of the lipid profile of a single lens worn for short time periods (15 minutes) as well as those worn for much longer periods. This is important due to the need to analyse the components involved in the early stages of spoilation as well as those involved in the later stages. The use of the fluorescence spectrophotofluorimeter enabled the biological components which adhere to the contact lens to be assessed non-destructively, the lens could then be returned to the ocular environment for further wear. Thus, the build-up of biological material *in vivo* and *in vitro* could be monitored as it accumulated on the surface of the lens.

The results of the high performance liquid chromatography (HPLC) yielded previously unavailable valuable information about variations which occur in individual tear chemistry. The ability to analyse these variations and produce patient tear profiles is a major achievement. All previous analytical studies used several contact lenses from unknown sources. From over two hundred chromatograms, left and right eye examination shows that the lipid profiles are usually very similar when obvious 'flyers' were omitted. These 'flyers' were attributed to other factors, for example, lid movement over spoilation phenomena producing over stimulation of the Meibomian glands or diseased states.

Analysis of the lenses studied indicated the presence of the following lipid classes cholesterol esters, triglycerides, fatty acids, cholesterol and probably diglycerides, monoglycerides, fatty alcohols and phospholipids. A number of variations occur in the quantity of lipoidal material extracted from the single contact lenses studied. The contact lenses worn for short time periods had a much larger number of components of greater concentration than those extracted from deposited lenses. The nature of the spoilation also appears to alter the lipoidal composition of the deposit. This was particularly noticeable with the calcium film lenses which possessed a high proportion of cholesterol esters with low levels of fatty acids, triglycerides and cholesterol. The contact lenses with and without discrete elevated deposits possessed a higher proportion of triglycerides, fatty acids and cholesterol than the contact lenses from which they were excised. A proportion of lipids present on the lenses may have been the result of handling the lenses during cleaning and insertion etc., although in the initial stages of spoilation it is likely that the lipoidal mass of the tears is much greater than the skin lipids. One interesting result of these high performance liquid chromatography (HPLC) studies was the low proportion of fatty acids which were extracted from the deposited lenses. This may be due to lipid penetration into the polymer network or surface polymerisation.

An *in vitro* model was also developed which mimics the important early stages involved in the spoilation process. The surrogate tear solution used being based on diluted foetal calf serum whose lipoidal composition is fairly similar to tears, particularly for the fatty acid family. This *in vitro* model enabled a variety of factors which may be involved in spoilation to be investigated. These factors included the effect of hydrogel composition; the effect of particular tear components, for example, lysozyme; the effect of wear schedule and the effect of cleaning regimes. As a result of these studies a number of conclusions can

be drawn. These include:-

1. Lysozyme is not vital to the spoilation process as surrogate tear components were still deposited onto the contact lens surface in its absence. It's deposition does, however, show more marked sensitivity to surface charge than other tear components.

2. Alteration of hydrogel composition by the addition of methacrylic acid, as explained in this context, changes the initial spoilation process. A small amount of negative charge proved beneficial and increased biocompatibility. Increased levels, however, attracted a greater level of deposition to the lens surface

3. The surfactant and enzyme cleaners are only partially effective. Enzyme cleaners appear imperfect in that enzyme fragments are produced during cleaning which may also be involved in spoilation; the specificity of the enzymes to certain proteins e.g. lactoferrin is variable and that a proportion of enzyme is adsorbed onto the contact lens surfaces, even without exposure to either *in vivo* or *in vitro* tears. This means that the progression of spoilation maybe affected by the cleaning regime employed and also highlights the difficulty in distinguishing the extrinsic from intrinsic factors.

In addition to the above conclusions, a number of other points arose during the use of the *in vitro* model. A residual fluorescence intensity is observed after extraction and cleaning of spoilt contact lenses which is probably the result of lipid penetration into the polymer network. This type of penetration is common with lipids, sugars, steroids and proteins, where the polymer network is sufficiently porous to allow large molecule penetration [244]. HEMA based hydrogels will readily allow the diffusion of lipids into the matrix, particularly the sodium salts of the free fatty acids. It is also likely that as these lipids become fractionated and separated from the lipid antioxidants which are present in the

eye [245], polymerisation will occur. This would also account for the build-up of 'sheets' of biological components on the contact lens surface which are observed during *in vitro* spoilation. These 'sheets' have a finite mass prior to being lost from the contact lens surface. This was illustrated by the decrease which was observed in the fluorescence intensity of the contact lenses after several days exposure to the model. The *in vitro* analogy has no lid motion pattern to produce mechanical disturbance of this biofilm on the contact lens surface. The deposit build-up observed whilst chemically mimicking the ocular environment has a lower frequency cycle, which accounts for this build-up and loss pattern observed. The progression of spoilation appears to be fairly rapid initially before reaching an equilibrium where further increases in the fluorescence intensity are smaller.

Although surface alterations and the amphoteric nature of the components used in the model affect the intensity of the spoilation peak observed, both it and the spoilation rate for different lens materials are remarkably similar in the early stages of spoilation i.e. within a thirty day period they are almost equal (i.e. within double the fluorescence intensity). This suggests that tear chemistry is probably more important than lens chemistry, within certain limits, in producing spoilation. It would not be true, however, for extreme lens compositions. This effect of tear chemistry is also supported by *in vivo* results. The early stages of spoilation are, therefore, likely to be tear derived and the later stages probably involve other extrinsic factors such as cleaning regimes, bad lens hygiene, poor blinking, exposure to UV light etc..

The formation of areas of localised drying out of the tear film on the lens maybe the initiating factor in lipid deposition. These dry spots form due to thinning out of the tear

film, so that lipid migrates, contaminating the mucous surface , creating a non-wettable area [160]. Thinning may occur on the surface of the contact lens, due to inefficient blinking or a decreased tear flow, allowing the lipid layer of the tear film to contact the lens surface. The composition of the hydrophilic contact lens surface would allow the carboxyl groups of the fatty acids to adhere loosely to the polar groups present in the matrix. This is thought to be one possible mechanism to allow lipid adsorption onto the contact lens. The total mechanism by which these lipids form white spots on the lens surface is unclear. It is, however, thought that adsorbed unsaturated fatty acids may polymerise to these localised areas of polymerised lipid on the lens surface. The high degree of unsaturation observed at the lens-deposit interface, as compared to the rest of the deposit [140] suggests that this process does occur. It also seems certain that once lipid adsorption onto the lens surface has occurred its surface properties are altered thus enabling further tear component adsorption onto the lens to occur more easily.

In addition to the conclusions drawn from the *in vitro* model it is further concluded that unsaturated tear lipids, probably unsaturated fatty acids, are involved in the interfacial conversion process of hydrogel lenses, reducing their compatibility with the ocular microenvironment. Lipid interaction with the lens surface then facilitates secondary deposition of other tear components. Interaction, exchange and immobilisation (by polymerisation) of the lipid layer being a key preliminary factor for the final and rapid growth of more complex, insoluble discrete deposits. Two major contributions of the studies devised here to the understanding of ocular spoilation are the development of analytical techniques sufficiently sensitive to investigate the minute quantities of biological components on a single contact lens and the development of an *in vitro* model which

mimics the early stages of the interfacial conversion process. The further use of these techniques in conjunction with clinical studies will provide a sound scientific basis for the study of ocular spoilation.

Results obtained in the course of these studies indicate the need for the application of further analytical procedures to the deposition processes. Other analytical techniques which may prove useful in aiding the identification of the lipoidal components, for example, include gas chromatography - mass spectrometry (GC-MS), fluorescence tagging and multiple wavelength high performance liquid chromatography. The effect of other criteria on the spoilation process also needs to be investigated. For example, the effect of ultra-violet (UV) light and oxygen which may cause auto-oxidation of the lipoidal species leading to polymerisation and olgiomerisation on the surface of the contact lens, plasma etching of the lens surface immediately prior to use and the effect of inadequate blinking on the chemistry of spoilation could also be studied with advantage.

A number of extrinsic factors also need further investigation, these might include the effect of cosmetics and the use of 'good' and 'bad' care regimes. It will also be valuable to carry out parallel *in vivo* and *in vitro* studies using the same lens materials and care systems, in order to make more absolute comparisons of the initial rates of deposition in the eye and in the tear model.

In summary, analytical techniques lie at the heart of successful studies of the multiple effects that contribute to the phenomenon of biocompatibility. It is hoped that this thesis advances the quantitative analytical basis that is necessary for the study and greater understanding of processes involved in ocular spoilation.

APPENDIX 1.

Formulations of fixatives and buffers used in this study.

Fixatives.

10 Percent Formalin.

100mls concentrated formaldehyde solution (37-40 percent, B.D.H.), 90mls of freshly distilled water.

Formal-calcium.

1 percent (w/v) $CaCl_2$ in neutral 10 percent formaldehyde.

Neutral Buffered Formaldehyde solution.

100mls 37 percent formaldehyde, 900mls distilled water, 4g acid sodium phosphate monohydrate ($Na_2H_2PO_4H_2O$), 6.5g anhydrous disodium phosphate (Na_2HPO_4).

Neutral 10 Percent Formaldehyde.

100mls formaldehyde solution (37-40 percent), 900mls distilled water, calcium carbonate (chips) in excess.

Buffers - Phosphate buffered saline.

Stock solution.

A - 0.2M solution of monobasic sodium phosphate, NaH_2PO_4 (27.58g/100ml)

B - 0.2M solution of dibasic sodium phosphate, Na_2HPO_4, (28.38g/100ml)

pH 7.4 95mls of solution A plus 405mls of solution B; add 500mls of distilled water and 9.5g of NaCl.

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